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Peroxisome proliferator-activated receptor gamma (PPARγ): linking peripheral metabolism with stress-related anomalies in the mouse brain

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"Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution. It is, strictly speaking, a real factor in scientific research."

— *Albert Einstein*

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Abstract

Cognition, emotion and mood are interrelated behavioural domains that are deleteriously affected by stress. Stress is also causally related to metabolic disorders such as obesity and diabetes, both of which are associated with an increased risk to develop mood and cognitive impairments, including severe forms of the latter such as Alzheimer's disease (AD). The work in this thesis aimed to explore a mechanism likely to link these various pathological states. The studies, carried out in mice, focussed on the peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated nuclear receptor that is a key regulator of adipocyte differentiation, lipid storage and glucose metabolism; moreover, PPARγ agonists are potent insulin sensitizers. Recently PPARγ agonists, namely thiazolidinediones (TZDs), have been proposed as therapeutic agents for a variety of brain disorders, including AD. In addition, some studies have implicated PPARγ in the regulation of the physiological response to stress.

At present, it is not clear as to whether TZDs act directly in the brain or whether their effects represent indirect actions on glucose metabolism. To this end, an attempt was made to map the expression of PPARγ mRNA and protein in mouse brain, focusing on areas involved in the regulation of cognition, feeding and endocrine function; comparisons were made between brains from control mice and mice exposed to high-fat diet (HFD) to the point of obesity since obesity is known to regulate peripheral levels of PPARγ. Results of these experiments suggest that PPARγ is indeed expressed in mouse brain under basal conditions, albeit at very low levels that can be slightly upregulated by HFD. More definitive answers regarding the question of centrally-expressed PPARγ awaits the development of improved reagents, in particular more specific antibodies.

In light of the link between stress, metabolic disturbances and AD, one of the experiments reported here investigated whether pioglitazone, a potent TZD PPARγ ligand, can modulate stressed-induced metabolic and cognitive dysfunction. While stress predictably impaired glucose tolerance and insulin sensitivity, reduced body weight, increased locomotor behaviour, and altered regulatory set-points of the hypothalamic-pituitary-adrenal (HPA) axis, pioglitazone normalized stress-induced hyperglycemia, insulin insensitivity and body weight loss, but failed to reverse hyperlocomotion and produced changes in HPA axis that varied according to specific test conditions. Furthermore, pioglitazone produced bidirectional effects on hippocampus- and fronto-cortical-

dependent cognitive behaviours and significantly reduced motivation and appetitive learning when food was the rewarding stimulus. The latter results, to some extent, help explain the apparently paradoxical actions of TZDs on insulin sensitivity and body weight. Immunoblotting analysis of hippocampal and frontal cortical tissue confirmed previous observations that, in specific brain regions, stress increases the levels of tau (an AD-related protein) and of hyperphosphorylated forms of the protein, which serve a neuropathological hallmark of AD; interestingly, although pioglitazone failed to reverse the stress-induced changes, it significantly reduced the levels of hyperphosphorylated tau, in the dorsal hippocampus and cortex of control animals.

Feeding behaviour strongly depends on motivation and cognitive processes such as learning, memory and decision-making, all of which are disturbed in AD. Extending recent work by others showing that PPARγ agonists reduce motivation for drugs and substances of abuse, the present research revealed that pioglitazone reduces motivation for, and appetitive learning of, food rewards. Going further, an attempt was made to examine whether pioglitazone affects the general motivational state of mice or specifically the motivation for energy-related (food) rewards. Our experiments showed that the effects of chronic treatment with pioglitazone (6 weeks) on motivation and operant learning depend on the subject's body weight and energetic needs. This work was subsequently complemented with a test of hedonic preference, as a means to gain further insight into the role of PPARγ in regulation of the reward pathway, and therefore, food consumption and body weight. To this end, mice were given the choice between sucrose (sweet, energy-rich), saccharin (sweet, energy-free) and water in a sated or fasted state. While both groups showed a strong preference for saccharin, this preference, was markedly decreased by pioglitazone in fasted state, indicating that pioglitazone is a potential modifier of hedonic eating.

In summary, the results presented here strongly suggest that PPARγ might link stress, peripheral metabolism and cognitive function, although the underlying mechanisms remain unclear. Although the work did not resolve the question of whether TZDs exert their purported cognitive-improving effects directly in the brain, indirectly through their improvement of peripheral metabolism, or a combination of both, the results strongly support efforts to explore the potential benefits of targeting PPARγ in order to delay, improve or indeed reverse the behavioural impairments found in AD.

List of abbreviations

AB	Aminoterminal domain
Aβ	Amyloid beta
ABCA1	ATP-binding cassette transporter A1
AD	Alzheimer's disease
Acb	Nucleus accumbens
ACTH	Adrenocorticotropic hormone
ACBP	acyl-CoA–binding protein
ACS	acyl-CoA synthetase
AF2	Activation function 2
AgRP	Agouti-related protein
αP2	Fatty acid binding protein 2
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Arc	Arcuate
ATP	Adenosine triphosphate
AUC	Area under the curve
AVP	Arginine vasopressin
11β-HSD1 or 2	11β-hydroxysteroid-dehydrogenase type 1 or 2
BACE-1	β -site APP cleaving enzyme
BBB	Blood brain barrier
BDNF	Brain-derive neurotrophic factor
BKO	Brain knock out
BNST	Bed nucleus of the stria terminalis
BAT	Brown adipose tissue
BW	Body weight
CA1 or 2	*Cornu ammonis* area 1 or 2
CBP/p300	CREB-binding protein/ adenovirus early region 1A binding protein p300
C83	83-residue C-terminal fragment
C99	99-residue C-terminal fragment
CD36	Cluster of differentiation 36
C/EBPα	CCAAT/enhancer-binding protein α

CMS	Chronic mild stress
CNS	Central nervous system
CON	Control
CORT	Corticosterone
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding protein
CRH	Corticotropin-releasing hormone
CUS	Chronic unpredictable stress
DAB	Diaminobenzidine
DBD	DNA-binding domain
15d-PGJ2	15-Deoxy-Δ12,14-prostaglandin-J2
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
dH_2O	Distilled water
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
DR1	Direct repeat type 1
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENaC	Epithelial sodium channel
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
GABA	Gamma-aminobutyric acid
GC	Glucocorticoids
Glut4	Glucose transporter 4
GR	Glucocorticoid receptor
GSK3β	Glycogen synthase kinase 3β
GTT	Glucose tolerance test
GyK	Glycerol kinase
HFD	High-fat diet
9-HODE	9-hydroxy-10E,12Z-octadecadienoic acid
13-HODE	13-hydroxy-9Z,11E-octadecadienoic acid
HPA axis	Hypothalamic-pituitary-adrenal axis

HRP	Horseradish peroxidase
ICV	Intracerebroventricular
IDE	Insulin degrading enzyme
IHC	Immunohistochemistry
IL-6	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
ITT	Insulin tolerance test
IRS	Insulin receptor substrate
ISHH	*In-situ* hybridization histochemistry
L	Ligand
LBD	Ligand-binding domain
LH	Lateral hypothalamus
LPL	Lipoprotein lipase
LSD	Fisher's least significant difference
LXR	Liver X receptor
M1	Pro-inflammatory macrophages
M2	Anti-inflammatory macrophages
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
MAPK	Mitogen activated protein kinase
MEK	MAPK/ERK
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
msP	Marchigian Sardinian alcohol-preferring
N-CoR	Nuclear receptor co-repressor
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NFT	Neurofibrillary tangles
NHR/NR	Nuclear hormone receptor
NMDA	N-Methyl-D-aspartate
NOR	Novel object recognition task
NOS-2	Nitric oxide synthase 2

NPY	Neuropeptide Y
NSAIDS	Nonsteroidal anti-inflammatory drugs
N-terminal	Amino-terminal
OF	Open field test
O-GlcNAc	β-O-linked N-acetylglucosamine
OLR	Object location recognition task
PBS	Phosphate-buffered saline
PGC-1α	PPARγ coactivator-1α
PGE_2	Prostaglandin E2
PEPCK	Phosphoenolpyruvate carboxykinase
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PI3K	Phosphoinositide 3 kinase
PIO	Pioglitazone
POMC	Proopiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PRDM16	PR domain containing 16
PS-1 or PS-2	Presenilin gene 1 or 2
p-tau	Phospho-tau
PVN	Paraventricular hypothalamic nucleus
qPCR	Quantitative polymerase chain reaction
RIA	Radioimmunoassay
RMTg	Rostromedial tegmental nucleus
RT	Room temperature
RXR	Retinoid X receptor
sAPP	Soluble amyloid precursor protein
SC	Standard chow
SCh	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine

shRNA	Small hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SirT1	Sirtuin 1
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SPPARM	Selective PPARγ modulators
SRC	sarcoma tyrosine kinases
SSC	Saline-sodium citrate
SSRI	Serotonin reuptake inhibitors
STAT	Signal transducer and activator of transcription
STR	Stress
SUMO-1	Small ubiquitin-related modifier 1
TBS-T	Tris-buffered saline-Tween
T2D	Type 2 diabetes
TEA	Triethylamine
Tg	Transgenic
Thr	Threonin
TNFα	Tumor necrosis factor alpha
TRAP220	Thyroid receptor-associated protein complex 220 kDa component
tRNA	Transfer ribonucleic acid
TZD	Thiazolidinedione
UCP1 or 2	Uncoupling protein 1 or 2
VMH	Ventromedial hypothalamus
vs.	Versus
VTA	Ventral tegmental area
WAT	White adipose tissue

CHAPTER 1

General introduction

1.1 Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated nuclear receptors that belong to the family of nuclear hormone receptors (NHR or NR) which consists of at least 46 members, including estrogen, thyroid hormone and glucocorticoid receptors (Mangelsdorf *et al*., 1995). There are 3 PPAR-isoforms, coded by three separate genes; they are the PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) (Desvergne and Wahli, 1999; Willson *et al*., 2000; Harmon *et al*., 2011). All three isoforms are implicated in the regulation of lipid metabolism.

1.1.1 Structure and ligands

Like other NR, PPAR are potent transcription factors and consist of the 4 domains (**Figure 1.1**) typically found in other NR, namely a) an amino-terminal domain (A/B domain or N-terminal domain), which contains the ligand-independent activation function 1 (AF1) domain, b) a DNA-binding domain (DBD), c) a connecting hinge region and d) a carboxyl-terminal ligand-binding domain (LBD), containing the activation function 2 (AF2) domain. The DBD is highly conserved among all three PPAR types and consists of two zinc-fingers that bind to peroxisome proliferator response elements (PPRE) on PPAR target genes. The C-terminal LBD of PPAR, comprises 13 α-helices and a small 4-stranded β-sheet that is linked to the DBD by the hinge region. The PPAR ligand-binding pocket is rich in hydrophobic residues and is generally larger than that of other nuclear receptors. The C-terminal region also includes the AF2 domain, which provides a surface for interaction with co-activating or co-suppressors proteins that determine NR transcriptional activity. An important property of the C-terminal region is to allow heterodimerization of PPARs with another class of NR, retinoid X receptors (RXR); this dimerization is essential for the biological (transcriptional) activity of PPAR (Berger and Moller, 2002; Tontonoz and Spiegelman, 2008; Harmon *et al*., 2011; Sauer, 2015).

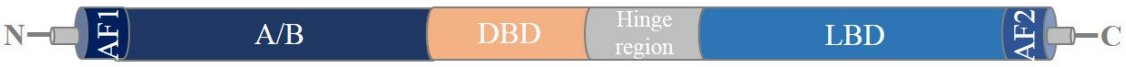


Figure 1.1. Structure of PPARγ receptor domains. A/B: amino-terminal domain, **AF1**: activation function 1 domain, **DBD**: DNA-binding domain, **LBD**: ligand-binding domain, **AF2**: activation function 2 domain.

Ligand activation leads to conformational changes in PPAR that promote their heterodimerization with 9-cis retinoic acid-liganded RXR (Berger and Moller, 2002). Subsequently, PPAR/RXR heterodimers bind to PPRE located in the promoter region of PPAR target genes where they initiate transcription (**Figure 1.2**). PPRE consist of direct repeat type 1 (DR1) sequences made up of two hexameric nucleotides with the consensus sequence AGGTCA that are separated by a single nucleotide (Willson *et al*., 2000; Berger and Moller, 2002; Harmon *et al*., 2011; Sauer, 2015).

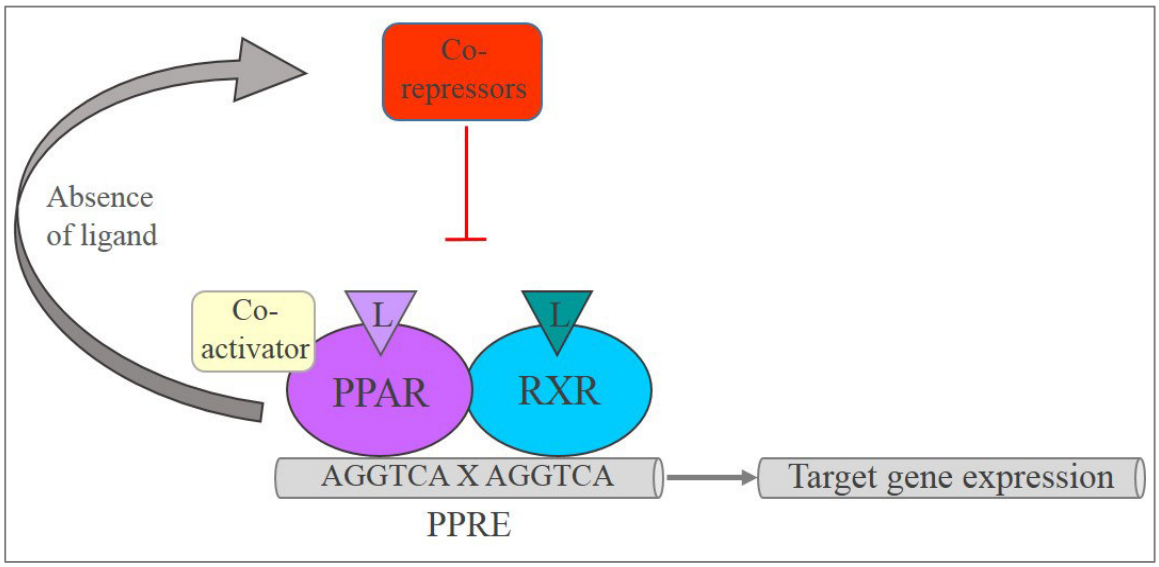


Figure 1.2. PPAR heterodimerize with RXR in the nucleus upon occupation by an appropriate ligand (L). Subsequent binding of the heterodimer to specific DNA sequences (peroxisome proliferator response elements, PPRE) leads to the transcriptional regulation of target genes. In the absence of the ligand, co-repressors hinder interactions of the unliganded receptor with the PPRE.

As shown in **Figure 1.2,** coactivators or co-repressors play an important role in modulating the transcriptional activity of, respectively, liganded and unliganded PPARγ. Major coactivator molecules include members of the CBP/p300 family, SRC family (sarcoma tyrosine kinases), TRAP220 and PPARγ coactivator-1α (PGC-1α) (Murphy and Holder, 2000; Tontonoz and Spiegelman, 2008), whereas the nuclear receptor co-repressor (N-CoR) and the silencing mediator for retinoid and thyroid hormone receptors (SMRT) represent key co-repressors of PPARγ function (Murphy and Holder, 2000; Tontonoz and Spiegelman, 2008; Lefterova *et al*., 2014).

Several natural and synthetic molecules have been identified to bind and stimulate or inhibit PPARγ. Natural PPARγ agonistic ligands include fatty acids and eicosanoids, as well as polyunsaturated fatty acids, such as linoleic acid, linolenic acid, arachidonic acid and eicosapentaenoic acid (Xu *et al*., 1999, Berger and Moller, 2002; Tontonoz and Spiegelman, 2008; Harmon *et al*., 2011; Sauer, 2015). Thiazolidinediones (TZDs or glitazones), represent an important class of synthetic PPARγ ligands; among these, rosiglitazone, troglitazone and pioglitazone (PIO), are compounds that have insulin-sensitizing properties and are therefore promising anti-diabetic agents (Hofmann and Colca, 1992; Nolan *et al*., 1994; Lehmann *et al*., 1995; Willson *et al*., 1996; Willson *et al*., 2000; Berger and Moller, 2002; Sauer, 2015). It should be noted that certain TZDs (e.g. KRP-297) have dual agonistic properties, also binding to either PPARα or PPARδ (Willson *et al*., 1996; Willson *et al*., 2000). Further, various non-steroidal anti-inflammatory drugs (NSAIDS), including indomethacin, fenoprofen and ibuprofen, are reportedly non-TZD PPARγ agonists (Lehmann *et al*., 1997; Sastre *et al*., 2006).

The activity of PPARγ is regulated by post-transcriptional modifications. Modifications such as mitogen activated protein kinase (MAPK)-mediated phosphorylation of PPARγ at Serine 112 (Hu *et al*., 1996), small ubiquitin-like modifier-1 (SUMO-1)-mediated sumoylation (Ohshima *et al*., 2004), and β-O-linked N-acetylglucosamine (O-GlcNAc)-mediated glycosylation (Ji *et al*., 2012) result in a reduction of PPARγ; further ubiquitinylation alters PPARγ activity (Kilroy *et al*., 2009) and importantly, deacetylation of PPARγ at Lysine268 and Lysine293 are important for "browning" white adipose tissue (WAT) into metabolically-activate brown adipose tissue (BAT) (Qiang *et al*., 2012).

1.1.2 Expression and function

PPARs play a critical role in lipid metabolism, but have been also implicated in other physiological and even behavioural functions.

PPARα is abundantly expressed in the brain, liver, gastrointestinal tract, kidney, heart, skeletal muscle, brown adipose tissue and various immune cell types (Braissant *et al*., 1996; Tyagi *et al*., 2011; Wahli and Michalik, 2012; Grygiel-Górniak, 2014). PPARα regulates fatty acid catabolism but has been also implicated in the inflammatory response and appears to reduce atherosclerosis and protect against coronary heart disease (Cho *et al*., 2008; Tyagi *et al*., 2011; Wahli and Michalik, 2012; Grygiel-Górniak, 2014).

The PPAR β/δ is the least-studied PPAR isoform. It is reported to be ubiquitously expressed and to contribute to fatty acid catabolism, glucose homeostasis and inflammation (Braissant *et al*., 1996; Tyagi *et al*., 2011; Wahli and Michalik, 2012; Grygiel-Górniak, 2014).

PPARγ, the subject of this thesis, occurs in 2 sub-isoforms (PPARγ1 and PPARγ2), both of which are found in humans (Fajas *et al*., 1997; Vidal-Puig *et al*., 1997) and rodents (Werman *et al*., 1997; Vidal-Puig *et al*., 1996). These isoforms result from alternative splicing, with PPARγ2 having an additional 30 amino acids at its N-terminus (Tontonoz *et al*., 1994a; Tontonoz and Spiegelman, 2008; Harmon *et al*., 2011; Ahmadian *et al*., 2013). A third isoform of PPARγ mRNA (PPARγ3) has been described in humans (Fajas *et al*., 1998). Although PPARγ3 derives as a product of an independent promoter, it codes for a protein identical to PPARγ1 (Fajas *et al*., 1998; Willson *et al*., 2000; Janani and Kumari, 2015) and is therefore usually simply referred to as PPARγ1 (Fajas *et al*., 1998).

PPARγ1 is widely expressed (e.g. in adipose tissue, heart, muscle, liver, gastrointestinal tract, kidney, pancreas and spleen), whereas PPARγ2 shows a more restricted expression pattern (mainly in adipose tissue and, at low levels, in muscle and liver) (Auboeuf *et al*., 1997; Fajas *et al*., 1997; Vidal-Puig *et al*., 1997; Willson *et al*., 2000). Both PPARγ isoforms are expressed at their highest levels in adipose tissue (Tontonoz *et al*., 1994a; Braissant *et al*., 1996; Auboeuf *et al*., 1997; Fajas *et al*., 1997; Vidal-Puig *et al*., 1997), where they regulate adipocyte differentiation, fatty acid storage and glucose metabolism (Lehrke and Lazar, 2005; Cho *et al*., 2008; Tontonoz and Spiegelman, 2008; Wahli and Michalik, 2012) (**Figure 1.3**).

Although PPARγ is widely studied in the field of cancer (including gliomas in brain), their role remains contradictory and their mechanisms of action unclear (Berger and Moller 2002; Michalik *et al*., 2004; Tontonoz and Spiegelman, 2008; Peters *et al*., 2012; Fröhlich and Wahl, 2015). Importantly, TZDs such as pioglitazone can reportedly increase the risk to develop bladder cancer (Cariou *et al*., 2012; Peters *et al*., 2012; Ahmadian *et al*., 2013; Soccio *et al*., 2014; Sauer, 2015) and have discouraged their application in diseases such as diabetes (Cariou *et al*., 2012; Soccio *et al*., 2014); however, it remains to be established to whether these unwanted effects reflect mediation of PPARγ or represent the inherent toxic properties of TZDs themselves. Other concerns that limit the therapeutic use of TZDs, is their induction of water retention (oedema) by

upregulating the epithelial sodium channel (ENaC) (Bełtowski *et al*., 2013; Fu *et al*., 2015); this condition is associated with cardiovascular stress which increases the risk of heart failure (Soccio *et al*., 2014; Pol *et al*., 2015). On the other hand, besides their insulin-sensitizing actions, activated PPARγ have beneficial immunomodulatory effects. For example, their activation by NSAIDS produce anti-inflammatory effects (**Figure 1.3**) by inhibiting the activation of inflammatory response genes (Pascual *et al*., 2005) in macrophages and regulating the polarization of pro-inflammatory macrophages (M1) into alternative anti-inflammatory macrophages (M2) (Bouhlel *et al*., 2007; Tontonoz and Spiegelman, 2008; Cariou *et al*., 2012; Wahli and Michalik, 2012). Furthermore, TZDs have been suggested to prevent neuroinflammation (see section 1.2.5), as pioglitazone treatment decreases the number of activated microglia and astrocytes as well as levels of pro-inflammatory enzymes in the hippocampus and cortex (Heneka *et al*., 2005).

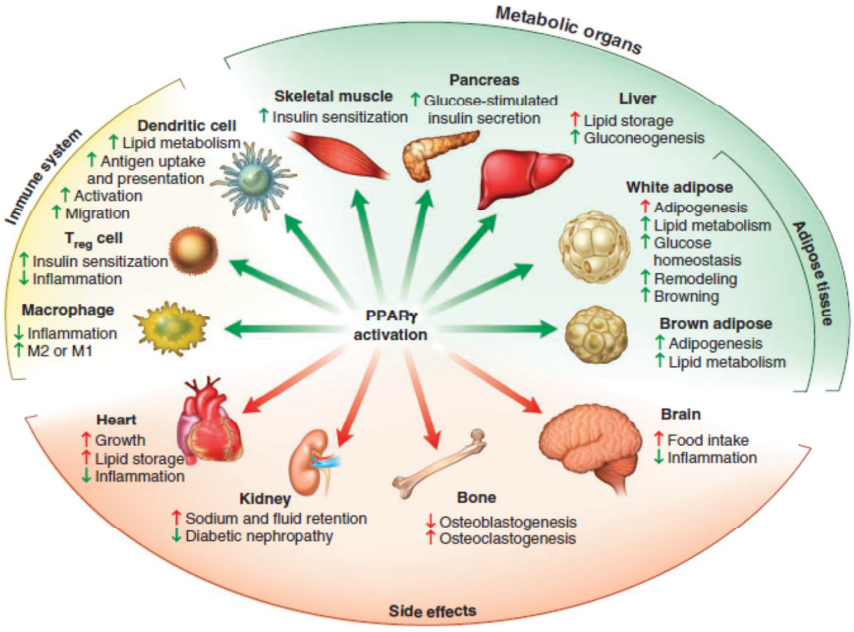


Figure 1.3. Diverse effects of PPARγ activation in specific tissues. PPARγ activation regulates primarily lipid and glucose metabolism but has been also shown to affect peripheral and central inflammation. Green arrows represent beneficial effects of the receptor's activation; red arrows show the side effects. *From:* **Ahmadian *et al*. 2013**.

In the context of the present work, it is important to mention that PPARγ have been detected in the developing rat brain (Braissant and Wahli, 1998) as well as in the adult rat brain and spinal cord (Braissant *et al*., 1996; Cullingford *et al*., 1998; Moreno *et al*.,

2004; Inestrosa *et al*., 2005; Cimini *et al*., 2005; Sarruf *et al*., 2009). In mice, there is some evidence for PPARγ protein expression by neuronal and non-neuronal cells in mice (Sarruf *et al*., 2009; Lu *et al*., 2011) and for the presence of PPARγ mRNA in the brain (e.g. neocortex, thalamus, hippocampus, amygdala, hypothalamus) (Liu *et al*., 2015). In our lab, PPARγ immunoreactivity and mRNA have been demonstrated in murine (postnatal day 4-5) hippocampal and frontocortical primary cultures, being mainly found in the neuronal subpopulation of cells (S. Moosecker, *unpublished).* Importantly, several studies suggest that central PPARγ can be regulated by peripheral manipulations such as fasting, high-fat diet (HFD) or peripheral administration of rosiglitazone (Diano *et al*., 2011; Garretson *et al*., 2015; Liu *et al*., 2015) and that TZDs can act directly upon brain PPARγ (Lu *et al*., 2011; Ryan *et al*., 2011; Denner *et al*., 2012). Notably, however, a major *in situ* hybridization histochemistry-based study on the distribution of nuclear hormone receptors in the adult mouse brain reported the absence of PPARγ gene in brain regions, the exceptions being the olfactory areas, cerebral cortex and cerebellum which expressed low levels (Gofflot *et al*., 2007). It should be noted however, that the type of screening method used does not allow for detailed cellular analyses or adjustment for assay sensitivity. Meanwhile, a number of authors have linked central PPARγ to neuronal cell differentiation and death as well as to neuroinflammation and neurodegeneration (Heneka and Landreth, 2007; Quintanilla *et al*., 2014). Both, animal and human studies have also described the therapeutic potential of TZDs in the treatment of cerebral ischemia and neurodegenerative disorders, such as Alzheimer's disease (AD) (also see section 1.2.5), Parkinson's disease and amyotrophic lateral sclerosis (Heneka and Landreth, 2007; García-Bueno *et al*., 2010; Zolezzi *et al*., 2014; Pérez and Quintanilla, 2015).

1.1.3 PPARγ: a key regulator master of glucose and lipid metabolism – role in periphery

PPARγ is known for its critical role in adipogenesis, adipocyte differentiation and fatty acid storage (**Figure 1.3**). Activation of PPARγ induces the transcription of target genes [e.g. CCAAT/enhancer-binding protein α (C/EBPα), fatty acid binding protein 2 (αP2), cluster of differentiation 36 (CD36), lipoprotein lipase (LPL), phosphoenolpyruvate carboxykinase (PEPCK), glucose transporter 4 (Glut4) and insulin receptor substrate 1 and 2 [(IRS-1, IRS-2)] that are involved in adipogenesis, lipid uptake and storage, and in

glucose homeostasis (Evans *et al*., 2004; Tontonoz and Spiegelman, 2008; Ahmadian *et al*., 2013; Lefterova *et al*., 2014) (**Figure 1.4**).

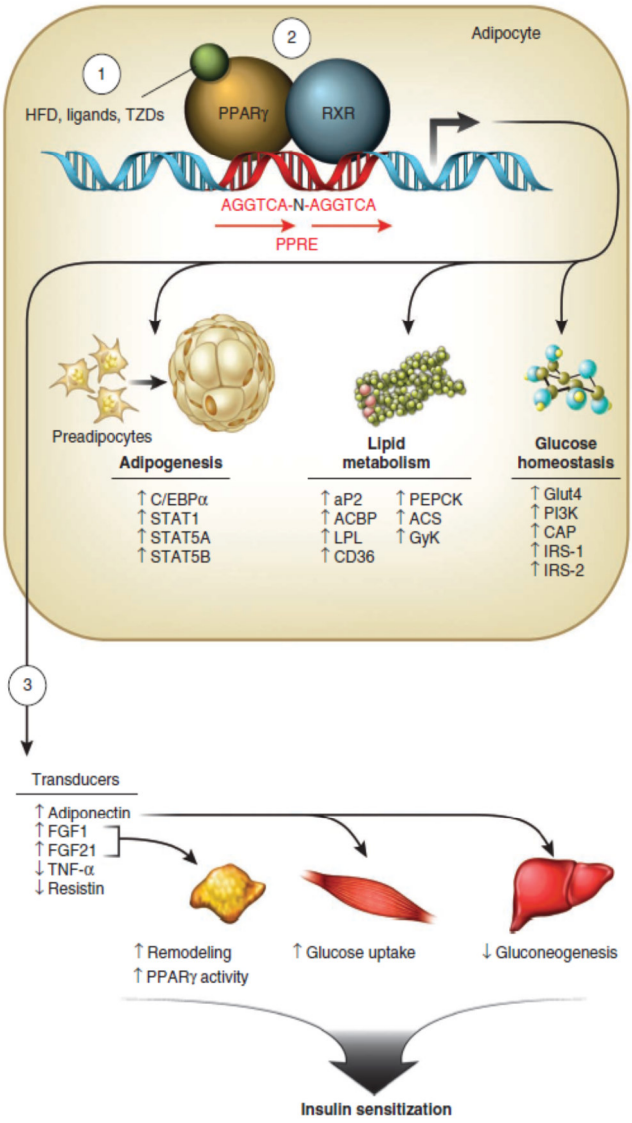


Figure 1.4. Effects of PPARγ in adipose tissue. *(Ahmadian et al., 2013).* Activation of PPARγ leads to its heterodimerization with RXR and subsequent activation of target genes which mediate adipocyte differentiation, lipid and glucose metabolism. Expression of the following key transducer molecules are regulated by PPARγ: *ACBP:* acyl-CoA–binding protein; *ACS:* acyl-CoA synthetase; *aP2:* fatty acid binding protein 2; *CD36:* cluster of differentiation 36; *C/EBPα:* CCAAT/enhancer-binding protein α; *Glut4:* glucose transporter 4; *GyK:* glycerol kinase; *IRS:* insulin receptor substrate; *LPL:* lipoprotein lipase; *PEPCK:* phosphoenolpyruvate carboxykinase; *PI3K:* phos-phoinositide 3 kinase; *STAT:* signal transducer and activator of transcription.

PPARγ stimulates adipocyte differentiation (Tontonoz *et al*., 1994b; Tontonoz *et al*., 1995) but is also required for the survival of mature adipocytes (Imai *et al*., 2004; Metzger *et al*., 2005). Mice lacking PPARγ cannot form adipose tissue (Rosen *et al*., 1999; 2002). Additionally, pharmacological inhibition of PPARγ by GW9662 protects mice from high-fat diet-induced obesity (Nakano *et al*., 2006). Indeed, studies in humans describe a role for PPARγ in obesity, with, for example, familial partial lipodystrophy (characterized by adipose tissue repartitioning and metabolic disorders, such as insulin resistance and dyslipidemia) being causally linked to heterozygous mutations in the

PPARγ gene (Hegele *et al*., 2002; Agarwal and Garg, 2002). Furthermore, human obesity correlates with higher levels of PPARγ gene expression (Vidal-Puig *et al*., 1997). Complementing these findings, work in rodents has shown that PPARγ1 and PPARγ2 mRNA levels in adipose tissue increase in mice made obese by exposure to a high-fat diet (HFD) (Vidal-Puig *et al*., 1996). Conversely, fasting decreases adipose tissue expression of both PPARγ1 and γ2 (Vidal-Puig *et al*., 1996).

As mentioned above, PPARγ agonists were recently shown to induce the transformation of white to brown adipose tissue (Ohno *et al*., 2012; Qiang *et al*., 2012) (**Figure 1.3**) and to increase the expression of uncoupling protein 1 (UCP1), which is critically involved in thermoregulation (Sell *et al*., 2004). Although the mechanism is still not clear, Qiang *et al*., (2012) suggested that deacetylation of PPARγ by SirT1 may be an important step in this process. The TZDs have an important role in regulating glucose metabolism; specifically, TZD activation of PPARγ in adipose tissue induces lipid uptake and storage as well as the expression of adipokines (e.g. adiponectin) that promote glucose uptake and inhibit the expression of molecules that induce insulin resistance (e.g. TNFα, resistin) (Evans *et al*., 2004; Tontonoz and Spiegelman, 2008; Ahmadian *et al*., 2013). Consistent with these observations, tissue- specific ablation of the PPARγ gene in skeletal muscle (Hevener *et al*., 2003; Norris *et al*., 2003) or liver (Matsusue *et al*., 2003) results in a diabetic phenotype (impaired glucose uptake and insulin resistance). Interestingly, mice lacking PPARγ in adipose tissue, display an insulin resistance restricted to fat and liver; these parameters are unaffected in muscle (He *et al*., 2003). The findings that gene polymorphisms in the ligand-binding domains of PPARγ1 and PPARγ2 associate with type 2 diabetes (T2D) (and hypertension) (Barroso *et al*., 1999) are consistent with all the other observations and suggest that PPAR play a key role in the treatment of metabolic disorders.

1.2 Stress: effects on mood and cognition

1.2.1 Physiology of stress

Stress may be defined as “a state in which homeostasis is actually threatened or perceived to be so” (Chrousos, 2009). Triggered by noxious endogenous or exogenous stimuli that are sensed and processed by a complex neural network in the central and peripheral

nervous systems, stress triggers physiological responses, that are orchestrated by the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis. In particular, parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) release the neuropeptides corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) which, in turn, stimulate the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the blood (**Figure 1.5**). The latter results in an increase in the synthesis and secretion of glucocorticoid hormones (GC; e.g. cortisol in humans, corticosterone in rodents) into the bloodstream from where they act on a variety of target tissues to mobilize glucose through the breakdown of hepatic glycogen stores, increase cardiovascular output, suppress reproductive and tissue regenerative functions and suppress immunity (Herman *et al*., 1996; de Kloet *et al*., 2005; Chrousos, 2009; Ulrich-Lai and Ryan, 2013).

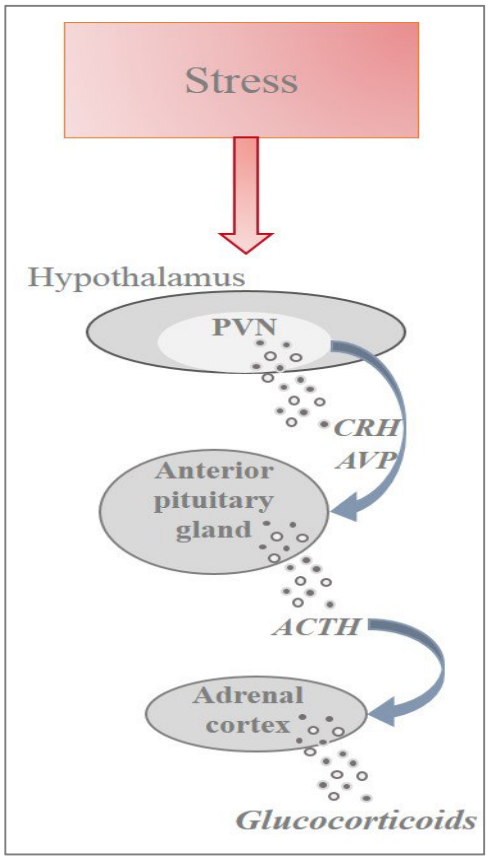


Figure 1.5. The endocrine response to stress is orchestrated by the hypothalamo-piuitary-adrenal (HPA) axis. Stress triggers the secretion of corticotropin releasing hormone (CRH) and arginine vasopression (AVH) from the hypothalamic paraventricular nucleus (PVN), which in turn stimulates the release of adrenocorticotropichormone (ACTH) from the pituitary and, subsequently, the synthesis and secretion of glucocortiocids (GC) such as cortisol and corticosterone from the adrenal gland.

The phasic nature of the stress response is depicted in **Figure 1.6.** In the first or "rapid" phase, hormones, such as catecholamines, CRH/AVP, neuropeptides and probably also GCs themselves, are released due to elevated GC levels. This leads to a fast response to stress, characterized by arousal and attention (de Kloet *et al*., 2005). The second, slower

phase of the stress response is usually characterized by a gradual turning off of the HPA axis, due to increased occupation of glucocorticoid (GR) and mineralocorticoid (MR) receptors by the secreted GC. These receptors are also transcription factors (members of the NR superfamily) and their liganded forms result in the induction or repression of genes that are responsible for the organism's long-term adaptation to the experienced stress. Both receptors are found ubiquitously but in the brain, GR are especially abundant in the hippocampus, an area in which MR are also abundantly expressed (Reul and de Kloet, 1985; de Kloet *et al*., 2005). Curtailing of the GC response to stress depends on negative feedback mechanisms primarily via the mediation of GR in the prefrontal cortex and hippocampus (Reul and de Kloet, 1985; Herman *et al*., 1996; Mizoguchi *et al*., 2003), two regions also strongly implicated in cognitive behaviours (executive functions and learning and memory). Other brain areas involved in the coordinating the hormonal response to stress are the amygdala and bed nucleus of the stria terminalis (BNST) which respond to stress by increased activity, i.e. counter the roles of the prefrontal cortex and hippocampus (Herman *et al*., 1996; Ulrich-Lai and Ryan, 2013).

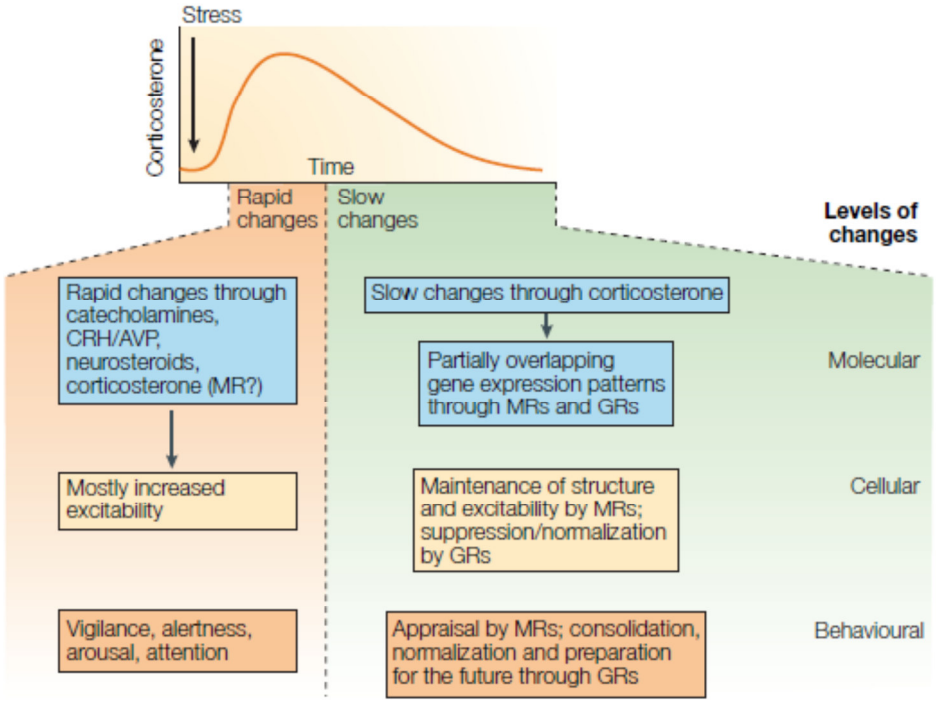


Figure 1.6. Rapid and gradual changes in response to stress. Elevated glucocorticoid (GC) levels lead to the secretion of hormones, including catecholamines, corticotropin releasing hormone (CRH)/arginine vasopressin (AVP), neuropeptides and probably also GCs, which induce the "rapid" stress response. Subsequently gradual changes are mediated by GCs action on specific genes. *From:* **de Kloet *et al*., 2005.**

1.2.2 Stress impairs mood and cognition

Normally, stress is a mechanism that contributes to the adaptation of the organism to challenges that disrupt its homeostasis. However, continuous exposure to increased GC levels (e.g. under conditions of chronic stress) can lead to detrimental effects on health, including hypertension, metabolic disorders (e.g. obesity, type 2 diabetes) but also synaptic loss, neuronal atrophy, that may be associated with mood disorders such as depression and cognitive decline that may be as severe as Alzheimer's disease (Sapolsky, 2000; Cerqueira *et al*., 2005 and 2007a; Sotiropoulos *et al*., 2008a; Catania *et al*., 2009; Chrousos, 2009; Sotiropoulos *et al*., 2011; Sousa and Almeida, 2012; Detka *et al*., 2013; Lopes *et al*., 2016; Sotiropoulos and Sousa, 2016). Many of these effects can be traced to the hippocampus with its abundant GR which, under these conditions also become impaired in their ability to downregulate HPA activity. Although the cellular pathways that mediate the effects on stress in the brain are still poorly known, several authors, have described stress-induced reductions in hippocampal and prefrontal cortical volumes (Sousa *et al*., 1998 and 1999; de Kloet *et al*., 2005; Cerqueira *et al*., 2005 and 2007a; Lupien *et al*., 2009; Detka *et al*., 2013) which can be mainly ascribed to atrophy of dendritic spines (Cook and Wellman, 2004; Cerqueira *et al*., 2007b). Notably, the hippocampus and prefrontal cortex are among the first to display the neuropathological hallmarks of AD (Braak and Braak, 1991; Sotiropoulos *et al*., 2008b; Serrano-Pozo *et al*., 2011; Braak and Del Tredici, 2015).

Many studies have suggested a link between glucocorticoids and the pathogenesis of AD, in particular because many AD patients show high levels of cortisol (Hartmann *et al*., 1997; Weiner *et al*., 1997; Rasmuson *et al*., 2001; Csernansky *et al*., 2006; Elgh *et al*., 2006; Sotiropoulos *et al*., 2008b). Our group previously reported that exposure to chronic stress or exogenous glucocorticoids, in rats, increases the pathogenic molecules that trigger AD, namely, increased levels of amyloid beta (Aβ, which results from the misprocessing of the amyloid precursor protein, APP), and abnormally hyperphosphorylated tau, which together disrupt memory, the characteristic complaint in AD patients (Sotiropoulos *et al*., 2008a; Catania *et al*., 2009; Sotiropoulos *et al*., 2011); similar findings were made in transgenic mouse models of AD (Green *et al*., 2006; Jeong *et al*., 2006). Aβ oligomers eventually form senile plaques and hyperphosphorylated tau form gives rise to neurofibrillary tangles, the two pathological hallmarks of AD (LaFerla and Oddo, 2005; Iqbal *et al*., 2010; Holtzman *et al*., 2011; Ittner and Götz, 2011; Selkoe

and Hardy, 2016; Sotiropoulos and Sousa, 2016). Interestingly, a recent study reported that an acute (single episode) stressor is sufficient to cause atrophy of dendritic spines in hippocampal neurons and to induce cognitive deficits in mice to an extent seen in AD-transgenic mice (Baglietto-Vargas *et al*., 2015).

1.2.3 Link between stress, metabolism and cognition

Impaired brain glucose metabolism, caused by stress has been suggested, together with insulin resistance, to link GC hypersecretion with cognitive and mood disorders (Detka *et al*., 2013). Interestingly, hypercortisolemia has been associated with insulin resistance and *vice versa* (Rasgon and Kenna, 2005). Animal studies have demonstrated hyperglycemia and insulin resistance to result from diverse chronic and acute stress paradigms (Zardooz *et al*., 2006; Depke *et al*., 2008; Rostamkhani *et al*., 2012; Ghalami *et al*., 2013; Li *et al*., 2013). In addition, long-term exposure to therapeutic dosages of GC have also been shown to induce hyperglycemia (Detka *et al*., 2013), the so-called clinical syndrome of steroid-induced diabetes mellitus (Hwang and Weiss, 2014). Here, it is worth noting that GC antagonize the actions of insulin to stimulate glucose uptake by triggering enzymes involved in gluconeogenesis (Detka *et al*., 2013; Hwang and Weiss, 2014).

There is growing evidence that insulin resistance/T2D contributes to the development of depression (Rasgon and Kenna, 2005; Detka *et al*., 2013) and cognitive disorders, including AD (Craft, 2007; de La Monte, 2009; Merlo *et al*., 2010; Luchsinger, 2012; Pérez and Quintanilla, 2015; Heneka *et al*., 2015b). Although not studied in this thesis, depression is mentioned because stress is one of the best known triggers of this condition (Patchev *et al*., 2014) and growing evidence suggests that depression may place individuals at risk for AD (Sotiropoulos *et al*., 2008b; Vyas *et al*., 2016; Kaup *et al*., 2016; Mirza *et al*., 2016); importantly, while there are no effective treatments for AD, certain antidepressants (selective serotonin-reuptake inhibitors, SSRIs) may exacerbate these mental disorders because of their tendency to induce weight gain (Rasgon and Kenna, 2005).

Recently, insulin resistance in the brain, has been increasingly recognized as a factor in causing cognitive disorders and even AD (Rasgon and Kenna, 2005; Detka *et al*., 2013; De Felice *et al*., 2014), thus AD has been proposed to be termed type 3 diabetes (de la Monte and Wands, 2008; de la Monte, 2014). Even though it is still unclear whether

peripheral and central insulin resistance have the same impact on brain functions (Jolivalt *et al*., 2008; Banks *et al*., 2012), this view is supported by work in insulin-deficient/diabetic mice that show decreased brain insulin signaling [and insulin degrading enzyme (IDE) expression] (Jolivalt *et al*., 2008; Merlo *et al*., 2010). These mice show Aβ deposition and increased amounts of abnormal tau hyperphosphorylation in their brains. Moreover, insulin is known to influence APP metabolism, its trafficking to the plasma membrane, and to modulate the release of Aβ into the extracellular space (where it exerts its neurotoxic effects) (Merlo *et al*., 2010). Interestingly, intranasal administration of insulin appears to improve cognition in AD (Banks *et al*., 2012).

Insulin resistance is often accompanied by hypertension and obesity, conditions that are risk factors for T2D. Further, consumption of fats and obesity (itself sometimes considered a stress-related disorder – Teegarden *et al*., 2008; Bose *et al*., 2009; Sanghez *et al*., 2013; Sominsky and Spencer, 2014; Razzoli *et al*., 2015; Razzoli and Bartolomuci, 2016) are significant risk factors not only for cardiovascular disease and diabetes but also for depression and severe cognition-impairing conditions such as AD (Rasgon and Kenna, 2005; Winocur and Greenwood, 2005; Farr *et al*., 2008; Smith *et al*., 2011; Nguyen *et al*., 2014). Studies in rodent models of obesity revealed impairments in memory and learning ability (Farr *et al*., 2008; Heyward *et al*., 2012; Valladolid-Acebes *et al*., 2013; Nguyen *et al*., 2014) that are reversible through a reduction of dietary triglycerides (Farr *et al*., 2008). Similar results have been observed in humans (Smith *et al*., 2011; Nguyen *et al*., 2014) with improvements being reported in individuals who lost weight (Smith *et al*., 2011). A correlation between obesity and AD pathology has been found in obese subjects that display increases in the levels of APP, Aβ, and total tau in the brain (Nguyen *et al*., 2014). A role for obesity in cognitive impairments is also suggested by data showing that overweight animals have smaller hippocampal volumes with concomitant signs of reduced neurogenesis, synaptic function and neuronal growth and reduced neuronal survival of hippocampal and hypothalamic neurons with parallel increases in brain levels of APP, Aβ, and tau phosphorylation (Nguyen *et al*., 2014).

1.2.4 Role of PPARγ agonists in the physiological response to stress

A number of studies have found an association between PPARγ and stress, suggesting that PPARγ signaling may be involved in the regulation of the physiological response to

stress. In particular, acute or repeated restraint stress in rats elevates cerebrocortical PPARγ protein expression (García-Bueno *et al*., 2005a, García-Bueno *et al*., 2008a) while adrenalectomy or inhibition of GC-synthesis or glucocorticoid receptor (GR) antagonism prevents the stress-induced up-regulation of PPARγ expression in the brain of rats (García-Bueno *et al*., 2008a). At present, the functional significance of this response to stress remains unknown, but it may serve to suppress undesired inflammatory responses since activated PPARγ reduces the expression of pro-inflammatory markers [Tumor necrosis factor alpha (TNFα), Nitric oxide synthase 2 (NOS-2), Cyclooxygenase-2 (COX-2)] (García-Bueno *et al*., 2005a,b; García-Bueno *et al*., 2008a,b), without affecting corticosterone levels, suggesting that the actions of PPARγ agonists in stressed brain are independent of their peripheral effects (García-Bueno *et al*., 2005b, García-Bueno *et al*., 2007). On the other hand, PPARγ agonist treatment does not reduce the secretion of adrenocorticotropic hormone (ACTH), the pituitary hormone that stimulates GC secretion (Ryan *et al*., 2012). Second, rats treated with PPARγ agonist rosiglitazone after stress-exposure display normalized glucose uptake and increased ATP levels in the brain compared to untreated animals that show impaired glucose metabolism and ATP levels in their brains (García-Bueno *et al*., 2007).

Other studies have also supported the view that rosiglitazone decreases the physiological responses to stress in rats (Ryan *et al*., 2012). Treatment with rosiglitazone reduced stress-induced heart rate- and the corticosterone response to stress. The drug also blunted neural activity (using c-Fos as a proxy marker) in the hypothalamic paraventricular and arcuate nuclei (Ryan *et al*., 2012). Additionally, Escribano *et al*., 2009 observed that rosiglitazone treatment improved cognitive deficits in AD-transgenic animals (overexpressing human APP), while lowering corticosterone levels by increasing GR expression in the hippocampus; these effects were most pronounced when mice were aged 10 months. The findings led the authors to suggest that PPARγ facilitate GC negative feedback, therefore helping to restore post-stress homeostasis.

Interesting studies by Matthews *et al*., (2009) provided a potential mechanism through which activated PPARγ might contribute to the regulation of GC secretion: these studies suggested that TZDs may be partial GR agonists. In particular, Matthews *et al*., (2009) showed that the TZD rosiglitazone (like the pure and potent GR agonist dexamethasone) induce GR phosphorylation at Serine 211, an event that leads to translocation of the receptor into the nucleus where it directs transcriptional activity. Despite the perplexing

finding that rosiglitazone triggered GR phosphorylation even in cells lacking PPARγ, the same authors showed that rosiglitazone potentiates anti-proliferative activity in cells overexpressing GR, suggesting that there might be actions of rosiglitazone which depend on GR. In an independent study, Ialenti *et al*., (2005) demonstrated that the TZD anti-inflammatory properties are GR-dependent, but PPARγ-independent; specifically, these investigators showed that in the absence of GR, TZDs fail to inhibit inducible nitric oxide synthase (iNOS) and IL-6 mRNA expression. Together, the reports by Ialenti *et al*., (2005) and Matthews *et al*., (2009), illustrate the complex relationship between PPARγ and GR in terms of ligand selectivity and signaling.

1.2.5 PPARγ agonists in stress related disorders: focus on Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder which represents 60-70% of the most common and severe form of dementia (Querfurth and LaFerla, 2010; Holtzman *et al*., 2011). The disease is characterized by progressive cognitive decline (primarily memory) (Querfurth and LaFerla, 2010). The deposition of aggregated amyloid beta (Aβ) and formation of neurofibrillary tangles, composed of abnormally hyperphosphorylated tau protein, and loss of forebrain cholinergic neurons represent the neuropathological hallmarks of AD (Wirths *et al*., 2004; LaFerla and Oddo, 2005; Holtzman *et al*., 2011; Sotiropoulos and Sousa, 2016). The Aβ peptides which form the characteristic extracellular plaques, are produced by the cleavage of the larger amyloid precursor protein (APP), a transmembrane protein (Wirths *et al*., 2004; Querfurth and LaFerla, 2010; Zolezzi *et al*., 2014). The APP processing includes two pathways: the amyloidogenic and non-amyloidogenic (**Figure 1.7**). In the amyloidogenic pathway, β-secretase (β-site APP cleaving enzyme; BACE-1) cleaves APP in the extracellular space to release a short sAPPβ fragment, and the remaining 99-residue C-terminal fragment (C99) is further cleaved by γ-secretase to yield 40 or 42 amino acid-long neurotoxic Aβ peptides. Non-amyloidogenic processing of APP involves α-secretase-mediated cleavage of APP to produce a soluble, secreted product (sAPPα) and the 83-residue C-terminal fragment (C83) which may be subsequently cleaved by γ-secretase to yield the short peptide, p3 (Wirths *et al*., 2004; Querfurth and LaFerla, 2010; Zolezzi *et al*., 2014).

Familial AD (or early-onset AD), with autosomal-dominant inheritance of mutations in the APP gene or in the presenilin 1 and 2 genes (presenilin is part of the γ-secretase complex), represents only <1% of all AD cases (LaFerla and Oddo, 2005; Merlo *et al*., 2010; Holtzman *et al*., 2011; Huang and Mucke, 2012; Liu *et al*., 2013). Sporadic AD (or late-onset AD) is by far more common, with aging being the greatest risk factor (Merlo *et al*., 2010; Holtzman *et al*., 2011; Pérez and Quintanilla, 2015). In addition, to obesity and T2D (Rasgon and Kenna, 2005; Winocur and Greenwood, 2005; Farr *et al*., 2008; Merlo *et al*., 2010; Smith *et al*., 2011; Luchsinger, 2012; Nguyen *et al*., 2014; Pérez and Quintanilla, 2015; Heneka *et al*., 2015b), carriers of just one ε4(E4) allele of the apolipoprotein E (ApoE) have a 40-80% risk of developing late-onset AD (Roses, 1996; Huang *et al*., 2004; Liu *et al*., 2013); the ApoE4 allele, which is linked to obesity and T2D because it causes disturbed lipid metabolism (Urosevic and Martins, 2008), has been implicated in dendritic spine loss, mitochondrial dysfunction and cognitive impairment (Brodbeck *et al*., 2008; Holtzman *et al*., 2011).

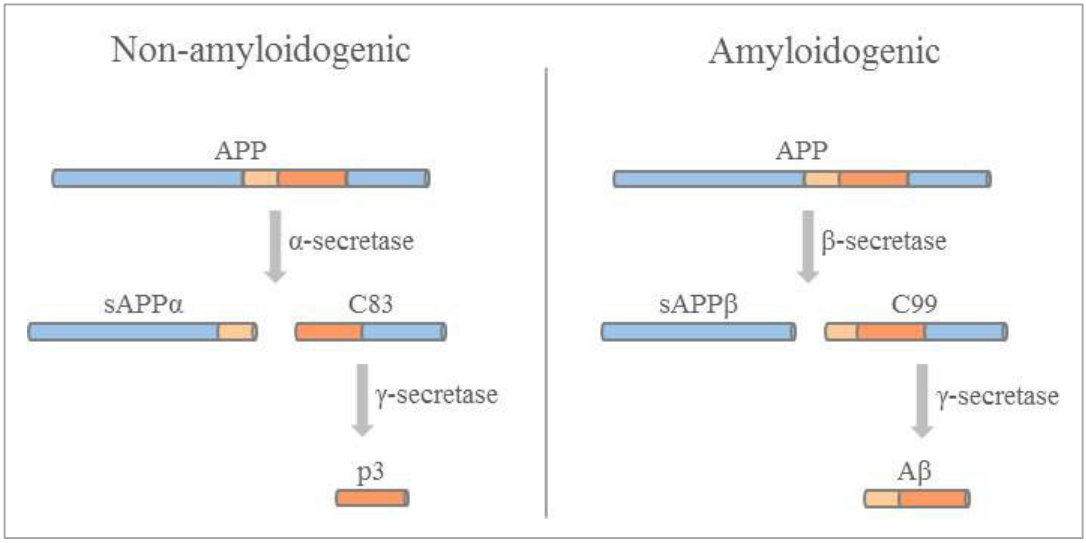


Figure 1.7. The amyloidogenic and non-amyloidogenic processing of the amyloid precursor protein (APP). In the non-amyloidogenic pathway, cleavage by the α-secretase produces the 83-residue C-terminal fragment (c83) and releases the sAPPα fragment; subsequent cleavage by γ-secretase results the short peptide called p3, thus precluding the production of Aβ peptides. The β-secretase involved in the amyloidogenic processing of APP, produces a short sAPPβ fragment and the 99-residue C-terminal fragment (C99) which is further cleaved by γ-secretase to generate 40 or 42 amino acid-containing neurotoxic Aβ peptides.

Tau is a microtubule-associated protein that binds and stabilizes microtubules (Lee *et al*., 2001) and is expressed in the central and peripheral nervous system (Gu *et al*., 1996; Lee *et al*., 2001). In the central nervous system (CNS), tau is most abundant in neurons and, to

a lesser extent, in astrocytes and oligodendrocytes (Lee *et al*., 2001). Tau has been reported to play an important role in synaptic plasticity (Hoover *et al*., 2010; Ittner *et al*., 2010; Kimura *et al*., 2010; Sotiropoulos *et al*., 2011; Kimura *et al*., 2013) and its hyperphosphorylation at specific serine and threonine sites by kinases such as glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (cdk5) leads to the so-called tauopathies, including AD (Lee *et al*., 2001; Takashima, 2006; Iqbal *et al*., 2010; Lei *et al*., 2011; Shukla *et al*., 2012; Papadopoulou *et al*., 2015).

PPARγ has been proposed as a therapeutic target for the treatment of Alzheimer's disease (AD), because of the ability of TZDs to ameliorate AD pathology. Most of the studies have been conducted in transgenic mouse models of AD (overexpressing human APP or human presenilin mutations), and demonstrated a TZD-induced (rosiglitazone) reductions in learning and memory deficits (Pedersen *et al*., 2006; Rodriguez-Rivera *et al*., 2011; Denner *et al*., 2012; Jahrling *et al*., 2014) or reduced Aβ42 peptide levels (but not amyloid plaque burden) in the brain (Pedersen *et al*., 2006). Similar effects of rosiglitazone were reported in a mouse model of AD which displays early cognitive deficits due to an APP (Swedish and Indiana mutations) transgene (Escribano *et al*., 2009; Escribano *et al*., 2010); the treatment reportedly produced a significant reduction in amyloid plaques and phospho-tau (p-tau) aggregates in the hippocampus (Escribano *et al*., 2010). Learning and memory improvements were observed when a PPARγ agonist was administered to mice expressing APP/PS1 transgenes (Mandrekar-Colucci *et al*., 2012; Toledo and Inestrosa, 2010; Chen *et al*., 2015), the behavioural changes being accompanied by decreases in Aβ levels and plaque pathology (Toledo and Inestrosa, 2010; Mandrekar-Colucci *et al*., 2012). Improvements in learning and reductions in Aβ deposits and tau pathology were also found when TZD treatments given to 3xTg-AD mice carrying a presenilin mutation while simultaneously, overexpressing APP and human tau (Searcy *et al*., 2012; Yu *et al*., 2015). Using yet a different transgenic mouse line (APPV717I), Heneka *et al*., (2005) and Sastre *et al*., (2006) found that TZDs and NSAIDS reduced Aβ levels and plaque pathology in middle-aged (10 months old) mice.

In vitro studies in both primary cells and cell lines have shown that PPARγ agonists reduce Aβ levels (Sastre *et al*., 2006; Mandrekar-Colucci *et al*., 2012), tau phosphorylation (Cho *et al*., 2013) and protect against Aβ-induced neurodegeneration (Inestrosa *et al*., 2005). Additionally, Brodbeck *et al*. (2008) showed that rosiglitazone dose-dependently increases dendritic spine density in rat primary cortical neurons and

prevents dendritic spine loss in cells carrying the ApoE4 mutation; the latter effect was shown to be mediated by PPARγ since it could be blocked with the PPARγ antagonist, GW9662.

In clinical trials, rosiglitazone was found to have positive effects in patients with mild-to-moderate AD. In one small study, rosiglitazone, but not placebo, over 6 months improved memory and cognition in patients with mild AD (Watson *et al*., 2005). Confirming this finding, a large phase II clinical trial in 600 patients with mild-to-moderate AD showed attention and memory improvements after 6 months treatment with rosiglitazone (Risner *et al*., 2006). Importantly, only non-ApoE4 carriers benefited from rosiglitazone treatment (Risner *et al*., 2006); this is important in view of the fact that subjects with PPAR-γ2 Pro12Ala polymorphisms are at greater risk for developing AD (Scacchi *et al*. 2007).

Application of TZDs in T2D patients was found to decrease the risk for dementia, when compared to metformin, a common medication for T2D (Heneka *et al*., 2015 b). Moreover, pioglitazone reduced cognitive deficits in patients with T2D and mild AD and improved insulin sensitivity in parallel (Hanyu *et al*., 2009; Sato *et al*., 2011). These findings indicate that PPARγ agonists modulate the course of AD pathology by virtue of their ability to improve insulin sensitivity not only in the periphery but possibly also in brain regions affected in AD. Studies in rat models of diabetes support this view, reporting improved memory and rescued glucose metabolism disturbances after TZD treatment (Yin *et al*., 2013; Fei *et al*., 2015; Ma *et al*., 2015). For example, TZD treatment was shown to be accompanied by improved insulin signaling in the hippocampus (Ma *et al*., 2015).

Other studies suggest that pioglitazone acts in a similar way to the NSAID ibuprofen to reduce Aβ load by downregulating BACE1 mRNA and protein levels as inhibiting activity of the BACE1 promoter (Heneka *et al*., 2005; Sastre *et al*., 2006). The observations of Heneka *et al*. (2005) and Sastre *et al*. (2006) were complemented by the finding that the prefrontal cortex of AD patients has markedly reduced levels of PPARγ protein (up to 40% less than in healthy subjects) that correlate negatively with BACE1 levels (Sastre *et al*., 2006). This finding was reproduced in APP-overexpressing (Tg2576) mice (Denner *et al*., 2012) who also showed that rosiglitazone treatment restores wildtype-like levels of PPARγ. With respect to the data suggesting that TZDs downregulate BACE1 expression, it should be mentioned that one study failed to detect

any effect of pioglitazone on BACE1 expression in the brain of 3xTg-AD (Searcy *et al*. 2012), raising doubts about this proposed mechanism of action. It should also be mentioned that non-TZD agonists of PPARγ (e.g. Astragaloside IV) in APP/PS1 transgenic mice reportedly downregulate BACE1 and thus, reduce Aβ levels and plaque burden in the brain (Wang *et al*., 2016).

An elegant set of experiments by Katsouri *et al*. (2011) suggested an interesting link between the PPARγ co-activator-1 α (PGC-1α) and AD pathology. These authors reported lower levels of PGC-1α in the AD brain; moreover, their *in vitro* studies demonstrated that overexpression of PGC-1α results in reduced activity of the BACE1 gene promoter and suppression of toxic Aβ peptide levels and their careful analysis revealed that all of these effects depend on the presence of PPARγ (Katsouri *et al*., 2011). Support for these results comes from the inverse correlation of PGC-1α expression levels and amounts of Aβ accumulation in APP transgenic mice as well as a cellular model of AD (Qin *et al*., 2009). In stark contrast, however, Dumont *et al*., (2014) reported that PGC-1α overexpression in a mouse model of AD elevates Aβ levels, tau deposition and neuronal death while further impairing cognitive performance. These conflicting sets of data regarding the role of PGC-1α in AD clearly warrant further exploration.

As mentioned before, TZDs have anti-inflammatory actions that are similar to those of NSAIDS. Consistently, TZDs were shown to rescue diabetes-induced activation of the nuclear factor κB (NF- κB) pathway and to decrease the overexpression of pro-inflammatory cytokines (Fei *et al*., 2015). Since neuroinflammatory mechanisms, including higher levels of microglial activation and recruitment of astrocytes to disease foci, have been increasingly implicated in AD pathology (Landreth and Heneka, 2001; Sastre *et al*., 2006; Heneka *et al*., 2015a), it is not surprising that several authors have followed the hypothesis that PPARγ agonists delay or reduce the extent of AD pathology through these pathways (it is thought that the pro-inflammatory molecules released by microglia might trigger neurodegeneration and cell death). In mouse transgenic models of AD, TZDs were shown to decrease microglial and astrocytic activation (Heneka *et al*., 2005; Mandrekar-Colucci *et al*., 2012; Papadopoulos *et al*., 2013) alongside reductions in hippocampal and cortical levels of pro-inflammatory enzymes (e.g. COX2, iNOS) with known neurotoxic functions (Heneka *et al*., 2005; Mandrekar-Colucci *et al*., 2012; Escribano *et al*., 2010). In addition, Xu *et al.* (2014) reported in rats that intra-cerebral rosiglitazone inhibits the increase of inflammatory cytokines induced by exogenous Aβ,

while providing protection against cognitive impairments associated with exposure to exogenous Aβ.

Recent work has proposed a role for activated PPARγ and its heterodimerization partner liver X receptor (LXR) in Aβ clearance. For example, pioglitazone was found to increase the expression of PPARγ and LXR as well as the transcription of PPARγ-LXR target genes such as ATP-binding cassette transporter A1 (ABCA1) and apolipoprotein E (ApoE) in APP/PS1 transgenic mice (Mandrekar-Colucci *et al*., 2012). These changes were accompanied by reduced Aβ levels and plaque load in the brain and the reversal of memory deficits. In another study, rosiglitazone was found to induce the expression of ABCA1 without affecting ApoE levels (Escribano *et al*., 2010). On the other hand, Searcy *et al*. (2012) reported an opposite (reduced) effect of pioglitazone on ABCA1 mRNA levels.

The previously-cited work by Denner *et al*. (2012) also showed that rosiglitazone improves cognition in an APP transgenic mouse line; this effect was PPARγ-dependent. The authors linked their observations to the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway which is known to play a critical role in hippocampus-dependent learning and memory (Atkins *et al*., 1998; Giovannini *et al*., 2015). Briefly, Denner *et al.* (2012) and Jahrling *et al.* (2014) found that rosiglitazone, acting through the mediation of PPARγ, increases the activity of ERK2 (Denner *et al*., 2012; Jahrling *et al*., 2014). Thus, these authors concluded that the ERK and PPARγ signaling pathways converge at some point. Other signaling pathways have also been implicated in an attempt to explain the pro-cognitive actions of TZDs. For example, following TZD treatment *in vivo*, Toledo and Inestrosa (2010) observed that activated Wnt signaling coincides with improvement of cognitive behaviour, reduced Aβ burden and fewer reactive glia. The same authors made similar findings in cultured rat hippocampal neurons (Inestrosa *et al*., 2005), and demonstrated that PPARγ agonists restore the loss of presynaptic and postsynaptic proteins in the hippocampus of APP transgenic animals (Toledo and Inestrosa, 2010). Many proteins other than ERK signaling also modulate synaptic plasticity and activity, one of the best studied being brain-derived neurotrophic factor (BDNF) the levels of which are also markedly reduced in AD patients. It is therefore interesting that central treatment of diabetic (db/db) mice with rosiglitazone corrected BDNF deficiency (Kariharan *et al*., 2015). The idea that PPARγ

agonists exert their positive effects in AD contexts by modulating synaptic function is reinforced by recent electrophysiological studies by Nenov *et al*. (2014; 2015).

1.3 PPARγ brain-periphery interplay

As mentioned previously (Sections 1.2.4 and 1.2.5), it is very likely that TZDs alter the course of AD because of their peripheral actions on glucose metabolism and/or their ability to maintain corticosterone levels within physiological limits. As an example, rosiglitazone was shown to reduce corticosterone levels (Pedersen *et al*., 2006; Escribano *et al*., 2009) and to reverse insulin resistance and impaired working and reference memory (Pedersen and Flynn, 2004) in AD transgenic mouse models; interestingly, inhibition of GC production by metyrapone had the same memory-restoring effects as rosiglitazone (Pedersen *et al*., 2006). Further, the latter authors reported that rosiglitazone ameliorates the decrease in insulin-degrading enzyme (IDE) in Tg2576 mice, possibly by countering the effects of high GC or by increasing insulin uptake by the brain (Pedersen *et al*., 2006). Other authors' work (Rodriguez-Rivers *et al*., 2011) support the view that rosiglitazone improves learning and memory deficits independently of its actions on glucose tolerance and hyperinsulinemia.

Although still unresolved, it would be too early to disregard the potential interplay between TZD-induced improvements in peripheral glucose homeostasis and cognition in light of strong evidence obtained in various mouse models of diabetes (Yin *et al*., 2013; Fei *et al*., 2015; Ma *et al*., 2015) as well as AD patients with diabetes (Hanyu *et al*., 2009; Sato *et al*., 2011). At the same time, it is notable that intracerebroventricular (ICV) treatment of diabetic mice with rosiglitazone reverses memory impairments without affecting peripheral measures of insulin sensitivity (Kariharan *et al*., 2015). The latter findings strongly imply that TZDs have a central site of action, a view supported by results of independent work by Denner *et al*. (2012) who showed that the pro-cognitive efficacy of rosiglitazone in Tg2576 mice is lost when central PPARγ is pharmacologically inhibited. Interestingly since rosiglitazone with/out PPARγ antagonism did not alter cognitive behaviour in wild-type animals, Denner *et al*. (2012) concluded that PPARγ does not have a role in regulating learning and memory processes in the absence of an underlying pathology. Additional evidence that rosiglitazone can act directly in the central nervous comes from the demonstration that memory deficits induced by Aβ42 in

rats are reversible by application of rosiglitazone directly into the dentate gyrus of the hippocampus (Xu *et al*., 2014).

It was mentioned that questions still remain as to whether PPARγ is expressed in the adult brain. An affirmative answer to this questions was provided by Ryan *et al*. (2011) who addressed the role of PPARγ in energy homeostasis in rats. These authors showed that rosiglitazone injections directly into the ventral hypothalamus or lentiviral-mediated overexpression of PPARγ in the hypothalamus stimulates higher food intake in association with gains in body and fat mass. Further, they reported that central PPARγ antagonism with GW9662 or shRNA-induced downregulation of brain PPARγ expression weakens the effects of rosiglitazone- or high fat diet (HFD) on food intake and body weight gain while also reversing HFD-induced leptin resistance (Ryan *et al*., 2011). Adding strength to the idea that the brain does express functional PPARγ, are the results from studies by Lu *et al.* (2011) who showed that brain-specific knockout of PPARγ (PPARγ-BKO) in mice results in decreased food intake, higher energy expenditure and thus, lower weight gain during exposure to a HFD. The PPARγ-BKO mice proved resistant to the hyperphagic effects of rosiglitazone and interestingly, to the insulin-sensitizing effects of rosiglitazone (Lu *et al*., 2011). Additional supporting evidence is provided by the observation that whole body or brain-specific deletion of the PPARγ coactivator-1α (PGC-1α) protects against diet-induced obesity in mice (Ma *et al*., 2010). Further, fasting and HFD, as well as peripherally-applied rosiglitazone upregulate hypothalamic levels of PPARγ mRNA (Diano *et al*., 2011; Liu *et al*., 2015; Garretson *et al*., 2015). Lastly, ablation of PPARγ specifically in proopiomelanocortin (POMC)-neurons, leads to increased energy expenditure, decreased food intake, lower body and fat mass, and higher brown fat mass, in HFD-maintained mice (Long *et al*., 2014). At the same time, the manipulation (POMC-PPARγ$^{-/-}$) improved glucose metabolism during HFD and neither agonism nor antagonism of peripheral PPARγ influenced food intake (Long *et al*., 2014). The anorexigenic POMC neurons are considered, together with the orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons, to be the main neuronal population in the arcuate nucleus (Arc) of the hypothalamus that are influenced by peripheral signals to regulate food intake (Sam *et al*., 2012).

All of the studies described above suggest the existence and role of brain PPARγ in the regulation of cognition and glucose/lipid metabolism. Interactions between the periphery and brain cannot be discounted, but at the same time, dissecting their individual

contributions to metabolic and behavioural homeostasis would appear to be an important, but difficult, objective. This becomes more interesting because of the known risk between obesity (along with its risk for T2D and other cardiometabolic disorders) and cognitive disorders such as AD. However, given that TZDs stimulate food intake and cause weight gain, another challenge is to examine the potential role of these antidiabetic compounds in the regulation of feeding behaviour; feeding is a primitive and simple behaviour that depends on cognitive processes such as learning and memory, all of which, in turn, depend on motivation. Emotion also plays an important role in feeding, learning/memory and motivation; however, although high GC (whose secretion is subject to regulation by PPARγ) generally have a negative impact on emotion, this behavioural dimension was not addressed in this thesis.

1.4 Involvement of PPARγ agonists in motivation and reward pathway

The nucleus accumbens (Acb) and ventral tegmental area (VTA) are key brain areas involved in the regulation of motivation, reward (and its anticipation and reinforcement) and pleasure (Fields *et al*., 2007; Richard *et al*., 2013; de Guglielmo *et al*., 2015; Berridge and Kringelbach, 2015; Castro *et al*., 2015). Dopamine neurons in the VTA, a midbrain structure, project to limbic areas, such as the Acb (core and shell), amygdala, hippocampus and medial prefrontal cortex (PFC; a center that, among others, coordinates executive functions) (Fields *et al*., 2007). In turn, the VTA receives inputs from the PFC, lateral hypothalamus (LH), bed nucleus of the stria terminalis (BNST, part of the so-called "extended amygdala"), and the superior colliculus. The LH also sends afferents containing the peptides orexin or α- melanocyte stimulating hormone to the VTA, and also innervates the PFC and amygdala. Many VTA projections to the Acb and PFC include GABA and glutamate as their transmitters (Fields *et al*., 2007). The VTA-Acb-PFC pathway, often referred to as the "mesocorticolimbic reward pathway" has been extensively studied in the context of addiction to drugs and substances of abuse since these are learnt appetitive behaviours.

Recent studies have implicated PPARγ signaling in modulation of the motivation and reward pathway, with TZDs being suggested as a new treatment for addictive disorders. Specifically, de Guglielmo *et al*., (2015) demonstrated that pioglitazone treatment

decreases heroin self-administration in rats by attenuating the rewarding properties of the drug and, therefore, the motivation to seek it; these effects were sensitive to a PPARγ antagonist. These authors also detected PPARγ in the posterior VTA, specifically in the rostromedial tegmental nucleus (RMTg) which is rich in GABAergic neurons and has an abundant population of opioid receptors which regulate dopamine transmission (Bourdy and Barrot, 2012). Pioglitazone was also shown to reduce alcohol consumption in an alcohol-preferring strain of rats (Stopponi *et al*., 2011; 2013), an effect that could be abolished by central administration of the PPARγ antagonist GW9662 (Stopponi *et al*., 2011). Together these findings show that activated PPARγ have a strong modulatory (inhibitory) influence on drug preference in animals, possibly by interfering with the motivational processes that underlie addictive behaviour.

In this thesis, interest in the role of PPARγ in the regulation of motivation stemmed from the somewhat counter-intuitive observations that TZDs increase insulin sensitivity although they stimulate food intake and cause increases in body (and especially white fat) mass in humans and animals (Lehrke and Lazar, 2005; Lu *et al*., 2011; Ryan *et al*., 2011 Cariou *et al*., 2012; Soccio *et al*., 2014). Accordingly, and in light of the reported effects of TZDs on motivation to retrieve pleasurable (hedonic) rewards, a large part of the studies in this work (Chapters 3 and 4) eventually focused on this question in an attempt to improve our understanding of the mechanisms of TZD action and their ability to link peripheral homeostatic events with centrally-regulated behaviours, such as eating. As will be described in Chapters 3 and 4, the tests used to examine this problem have a strong cognitive – learning/memory – component that could also inform on how TZDs exert their pro-cognitive actions.

1.5 Aims of the thesis

The review provided above demonstrates the paucity of studies on PPARγ actions in the brain, as well as the equivocal state of knowledge in this area. Accordingly, the specific aims of the present investigations were to

- determine the distribution of PPARγ in the mouse brain, with a focus on areas involved in cognition, neuroendocrine function and energy balance (**Chapter 2**);

- investigate the link between stress, metabolism, and cognition and their modulation by activation of PPARγ with pioglitazone (PIO), a potent TZD, in light of known cross-regulation between stress, cognition and metabolism (**Chapter 3**);

- examine the modulatory effects of TZDs on motivation to consume palatable foods and to acquire tasks based on appetitive learning (**Chapter 4**);

- attempt to develop a picture of how PPARγ contribute to the integration of peripheral and central signals which ultimately impact on cognitive behavior (**General discussion, Chapter 5**)

CHAPTER 2

Multiple approaches to detect PPARγ in the brain

Abstract

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear receptor that is a key regulator of adipocyte differentiation, lipid and glucose metabolism and, is strongly implicated in diabetes and obesity. Thiazolidinediones (TZDs), synthetic PPARγ agonists, were recently proposed as treatments for various neurological conditions, including Alzheimer's disease (AD). While the therapeutic potential of TZDs may lie in their anti-inflammatory and anti-oxidant properties, little is known about the mechanism of TZD actions in the brain; given the insulin-sensitizing activity of TZDs, and because metabolic disturbances are associated with AD pathology, it is possible that central TZD actions occur secondarily to their peripheral effects. To begin addressing this question, this study aimed at mapping the expression of PPARγ in the adult mouse brain. Our analysis focused on brain regions involved in the regulation of cognition and endocrine functions, with comparisons being made between brains from control mice and mice exposed to a high-fat diet (HFD); the reason for the latter choice is that PPARγ function is altered by obesity and obesity impacts on cognitive and endocrine functions. Since chronic stress has been implicated in obesity, diabetes and AD, brains from mice that had been simultaneously exposed to a chronic stress paradigm and TZD treatment were also analyzed for their content of PPARγ. Using a complementary mix of methods to detect PPARγ mRNA and protein, our results suggest that PPARγ is expressed at only low levels in adult mouse brain but which may, nevertheless, be subject to regulation by dietary manipulation.

2.1 Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated receptor, belonging to the nuclear receptor superfamily; its transcriptional actions depend on heterodimerization with retinoid X receptors (RXR) (Mangelsdorf *et al*., 1995; Berger and Moller, 2002; Tontonoz and Spiegelman, 2008; Harmon *et al*., 2011; Sauer, 2015). The PPARγ is a key regulator of adipocyte differentiation and is involved in lipid and glucose metabolism (Evans *et al*., 2004; Tontonoz and Spiegelman, 2008; Ahmadian *et al*., 2013). Two isoforms of PPARγ (PPARγ1 and PPARγ2) have been detected in peripheral tissues of humans (Fajas *et al*., 1997; Vidal-Puig *et al*., 1997) and rodents (Vidal-Puig *et al*., 1996; Werman *et al*., 1997). While the PPARγ1 isoform is expressed in adipose tissue, heart, muscle, liver, gastrointestinal tract, kidney, pancreas and spleen, expression of PPARγ2 is restricted to adipose tissue (high levels), muscle and liver (Auboeuf *et al*., 1997; Fajas *et al*., 1997; Vidal-Puig *et al*., 1997; Willson *et al*., 2000).

Thiazolidinediones (TZDs), which act as PPARγ agonists and serve as good anti-diabetic (insulin-sensitizing) drugs (Lehmann *et al*., 1995; Willson *et al*., 1996; Willson *et al*., 2000), have been suggested to be of therapeutic value for neurodegenerative disorders such as AD (Heneka and Landreth, 2007; Quintanilla *et al*., 2014). However, the extent (and spatial distribution) of PPARγ expression in the brain remains unclear. A number of studies have reported the presence of PPARγ in rat brain and spinal cord (Braissant *et al*., 1996; Cullingford *et al*., 1998; Moreno *et al*., 2004; Cimini *et al*., 2005; Inestrosa *et al*., 2005; Sarruf *et al*., 2009), including the developing rat brain (Braissant and Wahli, 1998). Further, Sarruf *et al*., (2009) localized PPARγ mRNA and immunoreactivity in both neuronal and non-neuronal cells, a result confirmed by Lu *et al.* (2011) who reported significant reductions in PPARγ mRNA expression in the hypothalamus, cerebral cortex and hippocampus (as well as brain stem, diencephalon and spinal cord) of mice in which the *PPARγ* gene was specifically deleted in neurons, as well as in non-neuronal cells in the CNS. Further, Diano *et al.,* (2011) reported that PPARγ in the mouse hypothalamus are regulated by high fat diet (HFD). More recently, Liu *et al*. (2015) reported widespread distribution of PPARγ in the mouse brain, with high levels of PPARγ mRNA expression in neurons (rather than glia) of the neocortex, olfactory bulb, the vascular organ of the lamina terminalis, and the subfornical organ. These authors also reported that certain subnuclei of the thalamus and amygdala, the choroid plexus and the hippocampus display

moderate levels of PPARγ mRNA but only low expression in all hypothalamic nuclei except for the suprachiasmatic nucleus, where fasting resulted in increased PPARγ mRNA expression levels. Notably, other authors observed low levels of PPARγ gene in olfactory areas, cerebral cortex and cerebellum but complete absence of PPARγ in other mouse brain regions (Gofflot *et al*., 2007). These sometimes divergent findings make it difficult to make a definite statement about central PPARγ expression and to exclude the possibility that PPARγ agonist effects in the brain reflect secondary effects in the periphery. Nevertheless, it is striking that intracerebroventricular injections (ICV) of rosiglitazone, a PPARγ agonist, were found to increase feeding behavior in rats (Ryan *et al*., 2011) and hamsters (Garretson *et al*., 2015). Additionally, it has been reported that ICV administration of rosiglitazone treatment restores memory deficits in diabetic mice (Kariharan *et al*., 2015). Furthermore, rosiglitazone administration normalizes synaptic activity and neuronal firing properties in hippocampal slices of transgenic animals (Nenov *et al.,* 2014; Nenov *et al*., 2015).

This study was undertaken in light of the equivocal reports regarding PPARγ expression in the adult mouse brain. In an attempt to resolve this issue, we used *in situ* hybridization histochemistry to detect PPARγ mRNA and immunochemical approaches (immunohistochemistry and Western blotting) to examine the spatial and quantitative distribution of PPARγ. These methodological approaches were applied to brains obtained from control and obese (HFD-exposed) adult mice, and from brains of adult mice that had been chronically stressed with/out co-administration of pioglitazone, a highly specific and potent PPARγ agonist; we focused on brain regions known to be involved in the regulation of cognitive and emotional behaviours as well as endocrinological and metabolic functions.

2.2 Materials and Methods

Animals

A total of 24 adult (3-4 months) male mice (Charles River Laboratories, Sulzfeld, Germany) were housed in groups (4 per cage) under standard animal housing conditions (22 °C, relative humidity 50 ± 10%), under a reversed 12 h light/12 h dark cycle (lights on at 17:00). Animals had *ad libitum* access to food and water, unless specifically

mentioned. Animal experiments were conducted in compliance with the European Union Council's Directive (2010/63/EU) and approved by the local commission for the Care and Use of Laboratory Animals of the State Government of Upper Bavaria. Two sets of experimental conditions were used in order to examine whether any PPARγ detected was subject to physiological regulation:

Control vs. obese mice: Mice received either a standard chow diet (SC, #1324 laboratory diet; Altromin, Lage, Germany) or high fat diet (HFD, D12451, ResearchDiets Inc., New Brunswick, NJ, USA) for 8 weeks.

Exposure to chronic unpredictable stress + PPARγ agonist: Tissues from animals used in the stress experiment described in Chapter 3 were used to determine PPARγ protein expression by immunoblotting analysis. Briefly, whereas the control group of animals were left undisturbed throughout, some animals were exposed to a chronic unpredictable stress (CUS) paradigm (see Chapter 3) for 6 weeks, followed by a chronic mild stress (CMS) for another 6 weeks. Throughout the experiment, half of each group (controls and CUS) received oral pioglitazone (Actos™ Takeda Pharma A/S, Denmark) in the SC diet (1324 laboratory diet, Altromin, Lage, Germany) at a dose of 20 mg/kg food (~ 3.3 mg/kg BW).

Intraperitoneal (i.p.) glucose tolerance test (GTT)

The GTT was performed in mice fasted for 16 h. To this end, resting blood glucose levels were measured before injecting them with 2 g/kg glucose i.p. (20% in saline; Sigma-Aldrich, St. Louis, MI, USA), after which blood glucose was again determined at 15, 30, 60 and 120 min. Glucose concentrations were measured using commercial glucometers (OneTouch® Vita®, LifeScan; Johnson and Johnson Medical, Neuss, Germany); blood samples were taken from a tail vein (small nick) of lightly-restrained mice.

Tissue collection

Animals were sacrificed by cervical dislocation at the end of each treatment paradigm, when their livers, visceral fat (epididymal) and brains were carefully excised, snap-frozen in isopentane (2-methylbutane; Sigma) and stored at -20° C until further analysis. A subgroup of mice was anaesthetized (sodium pentobarbital; Narcoren®; Merial, Halbergmoos, Germany) before intra-cardiac perfusion with 0.9% saline, followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS)[5]. Brains from these animals

were carefully removed, placed in 4% PFA (24 h), cryo-preserved (30% sucrose in PBS), and placed at -80° C until immunohistochemical analysis. In case of animals from stress experiments: animals were sacrificed by cervical dislocation 30 min after acute stress. The prefrontal cortex, hypothalamus, dorsal- and ventral hippocampus were dissected on ice and snap-frozen by placing in isopentane. Dissected brain areas were stored at -80°C until western blot analysis.

In-situ hybridization histochemistry (ISHH)

Serial coronal cryosections (10 µm thick) were cut from mouse brains, mounted onto SuperfrostPlus™ glass slides (Thermo Fisher, Braunschweig, Germany) and stored at -20° C before ISHH to detect PPARγ mRNA. All solutions used for pre-hybridization steps were prepared with diethylpyrocarbonate (DEPC)-treated water [5 ml DEPC (Sigma-Aldrich) in 5 L sterile water]. A PPARγ oligonucleotide[1] was 3' end-labelled with ^{35}S-dATP (Perkin Elmer, Waltham, MA, USA), purified with a QIAquick nucleotide removal kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Sections were pre-hybridized by fixation in 4% formaldehyde [in 0.01 M phosphate-buffered saline (PBS): 1:10 dilution of 0.1M PBS[2])], rinsed (2X) in PBS, and acetylated by incubation in 0.25% acetic anhydride (Sigma-Aldrich) in 0.1 M triethylamine/HCl [13.3 ml TEA (Sigma-Aldrich) and 6 ml 6N HCl, up to 1 L DEPC-water (pH 8.0)] for 10 min (RT). Sections were then dehydrated and serially delipidated in graded ethanol (EtOH) and chloroform (Sigma-Aldrich). Labelled probe was diluted (20.000 cpm/µl) in hybridization buffer[3]; 2 µl of 5 M 1,4-Dithiothreitol (DTT; Sigma-Aldrich) was added to 100 µl hybridization mixture and used to cover tissue sections. A glass coverslip was placed over the section/hybridization mix and slides were incubated (16 h) at 37° C, after which coverslips were removed by placing slides in 1x saline-sodium citrate (SSC) buffer (1:20 dilution of 20xSSC[4] in PBS) at room temperature (RT). Sections were then washed (4 x 15 min, 40° C) with a formamide/SSC solution (1:1 solution of formamide and 4xSSC). After two rinses in 1xSSC (RT, 30 min), sections were washed in distilled water

[1] **PPARγ oligonucleotide**: 5'-TGGAGTCCTCATCTCAGAGGGCCAAGGATTCATGACCAGGGAGTTCCTC (custom-synthesized by Sigma-Aldrich, using GenBank database at NCBI: http://www.ncbi.nlm.nih.gov/genbank/).

[2] **0.1 M phosphate buffered saline, 10xPBS** (pH 7.4): 90 g NaCl (Roth, Karlsruhe, Germany), 1.22 g KH_2PO_4 (Sigma-Aldrich-Aldrich), 8.15 g Na_2HPO_4 (Sigma-Aldrich), dissolved in 1 L DEPC-water.

[3] **Hybridization buffer**: 20 ml formamide (Merck, Darmstadt, Germany),8 ml 20xSSC[4], 2 ml salmon sperm DNA (Sigma- Aldrich), 0.4 ml yeast tRNA (25 mg/ml Sigma-Aldrich), 0.8 ml 50x Denhardt's solution (Sigma-Aldrich), 8 ml Dextran sulfate (Sigma-Aldrich), 0.8 ml sterile water.

[4] **20x saline-sodium citrate (20xSSC)**: 350.6 g NaCl, 176.4 g Tri-Na-citrate-dihydrate (Sigma-Aldrich-Aldrich), dissolved in 2 L DEPC-water.

(dH_2O) for 10 min, dehydrated in graded EtOH solutions (70-100%), air dried and exposed to a X-Ray film (BioMax MR film; Kodak, Rochester, NY, USA) for 2 weeks, at 4° C. Films were developed with Kodak Developer and Fixer (Sigma-Aldrich) and optically scanned (CanoScan 9950F, Canon Deutschland GmbH, Krefeld, Germany).

Immunohistochemistry (IHC)

Coronal cryosections from mouse brains (10 µm thickness) were cut, mounted on SuperfrostPlus™ glass slides and stored at -20° C until further processing. Sections were fixed for 5 min (4% formaldehyde in 0.01M PBS[5]) and washed 3 times in PBS before blocking endogenous peroxidases with 0.5% of hydrogen peroxide (Sigma-Aldrich) in PBS and 0.5% Triton X-100 (Sigma-Aldrich). After washing, an antigen retrieval protocol was applied by rinsing sections in sodium citrate buffer (2.94 g sodium citrate trisodium salt dehydrate in dH_2O, pH 6.0): incubation for 20 min at RT and for 30 min at 80 °C. After 2 rinses in PBS, sections were treated with blocking solution [2% normal goat serum (Sigma-Aldrich) in PBS; 1 h, RT] to reduce non-specific binding. Sections were subsequently incubated in primary antibody (overnight, 4° C); the antibodies used were listed in **Table 2.1**.

After washing (3x, 20 min in PBS), sections were incubated for 1 h, at RT with biotinylated secondary antibody (Vector Laboratories; BA-1000), diluted in blocking solution (1:300). After 3 washing steps (PBS, 10 min), sections were incubated in ABC working solution (Vectastain® Elite® ABC Kit; Vector Laboratories Inc., Burlingame, USA), according to manufacturer's instructions. Sections were then washed extensively in 0.01 M Tris (Sigma-Aldrich), pH 7.4, stained with diaminobenzidine (DAB) solution (DAB Substrate Kit for peroxidase; Vector Laboratories, Burlinghame, CA, USA), rinsed in tap water and washed (3x, 10 min) in Tris. Sections were then dehydrated in graded ethanol, cleared with xylene, and mounted in DPX mounting medium (Fluka Chemie AG, Buchs, Switzerland) before coverslipping. Sections were examined under a microscope, equipped with a camera and AxioVision Rel. 4.7 software (Carl Zeiss, Göttingen, Germany); images were imported and optimized (minimal sharpening an contrast) using Adobe Photoshop (Version 7.0).

[5] **0.1 M phosphate buffered saline, 10xPBS (pH 7.4):** 2 g KCl, 2,4 g KH_2PO_4, 80 g NaCl, 14,4 g Na_2HPO_4, dissolved in 1 L dH2O. **0.01M PBS (PBS):** 1:10 dilution of 0.1M PBS in dH2O.

Table 2.1. Primary antibodies used for analysis of PPARγ protein by IHC.

Antibody	Dilution	Company
Rabbit monoclonal anti-PPARγ (C26H12)	1:50	Cell Signaling; 2435
Rabbit polyclonal anti-PPARγ	1:100	Santa Cruz; sc-7196
Rabbit polyclonal anti-PPARγ	1:100	Abcam; ab19481

Immunoblotting

Mouse brains (cortex, hypothalamus, dorsal and ventral hippocampus) were analyzed for PPARγ immunoreactivity by Western immunoblotting. Proteins were isolated from frozen mouse brain areas by dounce homogenization in homogenization buffer[6]. After centrifugation (14.000 g, 4 °C, 15 min) lysates were aliquoted and stored at -80 °C until used. Protein concentrations were measured using Lowry's method (Lowry *et al*., 1951), with absorbance readings (750 nm) being made on a Synergy-HT reader (BioTek Instruments, Winooski, VT, USA). Known amounts of protein extract (40 µg) were then electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels to separate proteins according to their size. For this, samples were mixed with 6x Laemmli buffer[7], diluted in dH_2O to a volume of 20 µl and heat-denaturated (95 ºC, 5 min) before loading onto a 10% SDS-PAGE gel, along with a pre-stained molecular weight marker (Thermo Fisher). Electrophoresis was performed (75 V, 1.5-2 h) in an electrophoresis chamber (BioRad Laboratories, Hercules, CA, USA) filled with 1xSDS-running buffer (10xSDS[8] diluted 1:10 in dH_2O). Separated proteins were semi-dry transferred onto 0.2 µm nitrocellulose membranes (Trans-Blot Turbo Mini Nitrocellulose Transfer pack; BioRad) using the Turbo transfer system (BioRad) (2.5A, 25 V, 10 min). Membranes were stained with Ponceau-S Solution (Sigma-Aldrich) to assess the quality of protein transfer, washed in TBS-T (10xTBS-T[9] diluted 1:10 in dH_2O) and incubated (1 h, RT, with shaking) in blocking solution [5% non-fat milk powder (Roth) in TBS-T]. Thereafter, membranes were incubated (overnight, 4° C, with shaking) with primary

[6] **Homogenization buffer**: 100mM Tris (Sigma-Aldrich) pH 8.0, 1mM EDTA, 250 mM NaCl (Roth), 5 mM $MgCl_2$ (Sigma-Aldrich), 10% Glycerol (Sigma-Aldrich), 1% Nonidet P-40 (Fluka Chemie, Buchs, Switzerland), 20 µl of 50xProteinase inhibitor cocktail tablet (diluted in 10 ml dH2O; Roche, Mannheim, Germany), and 10 µl of (each) phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich).

[7] **6xLaemmli Buffer**: 3.5 ml 4xStacking gel buffer*, 1.5 ml Glycerol (Sigma-Aldrich), 0.465 g DTT, 0.5 g SDS (Sigma-Aldrich), 0.6 mg Bromophenol blue (Sigma-Aldrich), dissolved in 5 ml dH_2O (storage at -20°C). *_**Stacking gel buffer**_: 6.05 g Tris pH 6.8 and 0.4 g SDS, dissolved in 100 ml dH_2O.

[8] **10xSDS:** 30 g Tris (Sigma-Aldrich), 144 g glycine (ROTH), 1 g SDS in a volume of 1 L dH_2O

[9] **10xTris-buffered saline-Tween (10xTBS-T):** 1Volume 1M Tris pH 7.5-8.0, 1Volume 3M NaCl, 1% Tween-20 (ROTH), up to 2 L dH_2O.

antibodies (**Table 2.2**) diluted in blocking solution, except for the anti-PPARγ antibody which was diluted in 5% bovine serum albumin (Sigma-Aldrich) in TBS-T. Thereafter, membranes were washed extensively in TBS-T, and incubated (2 h, RT, with shaking) with a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (**Table 2.3**), diluted in blocking solution. Following this, membranes were again washed thoroughly in TBS-T and incubated with Lumi-Light Western Blotting Substrate (Roche). Proteins were visualized using a chemiluminescence reader (ChemiDoc MP Imaging System; BioRad) and semi-quantitative estimation of signal strength (at the correct band size of 50 kDa) was made using ImageLab 5.1 Software (BioRad).

Table 2.2. Primary antibodies used in immunobloting studies.

Antibody	Final Dilution	Supplier
Rabbit monoclonal anti-PPARγ (C26H12)	1:1000	Cell Signaling; 2435
Mouse monoclonal anti-Actin	1:5000	Chemicon; MAB1501R

Table 2.3. Secondary antibodies used in immunobloting analysis

Antibody	Final Dilution	Supplier
Goat anti-rabbit antibody (H+L) HRP-conjugated	1:2000	Cell Signaling; #7074
Sheep anti-mouse polyclonal antibody (H+L) HRP-conjugated	1:10000	Amersham; NA931

Statistical analysis

Statistical differences between groups were evaluated using Prism 6 software (GraphPad, San Diego, CA). After testing for normality of data, data comparisons between two groups were made using Student's *t*-test while multiple group comparisons were subjected to a 2-factor analysis of variance (2-ANOVA), followed by Sidak's or Bonferroni's multiple comparison (*post hoc*). The level of significance was set at $p < 0.05$; numerical data are presented as ± standard error mean (SEM).

2.3 Results

Efficacy of dietary manipulations

To determine regulation and specificity of brain PPARγ a subgroup of animals received high fat diet (HFD) to induce a state of overweight. The dietary regimens used to alter the expression of PPARγ were efficacious, as described below.

The two groups of mice were distributed initially so as to counterbalance their body weights (**Figure 2.1A**). Predictably, animals that received HFD displayed significantly ($p < 0.05$) higher body weights compared to controls by the end of the experiment (8 weeks exposure to HFD) (**Figure 2.1B**), their net gain of weight being significantly greater than that of control (SC-fed) mice during the same period ($p < 0.05$) (**Figure 2.1C**).

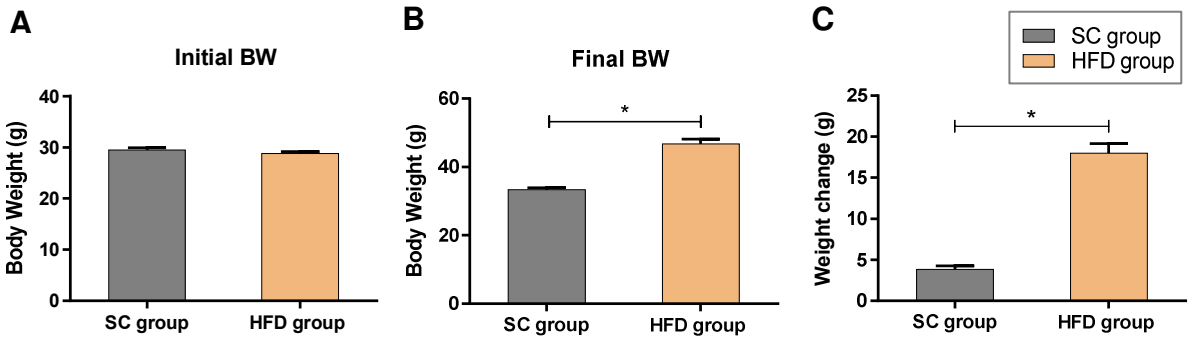


Figure 2.1. Analysis of body weight in mice before and after chronic exposure to a high fat diet (HFD). All animals were maintained on standard chow (SC) until the start of the experiment (16 weeks old). Thereafter, a subgroup of mice was placed on HFD for 8 weeks (n = 12), while another subgroup served as controls and was maintained on SC (n = 12). **(A)** Starting body weight in g; **(B)** Final body weight in g; **(C)** Weight change in g. Data are presented as ± SEM (standard error of mean), * $p < 0.05$.

Mice that had been placed on HFD displayed glucose intolerance when tested in the glucose tolerance test (GTT) at the end of the experiment, as shown in **Figure 2.2**.

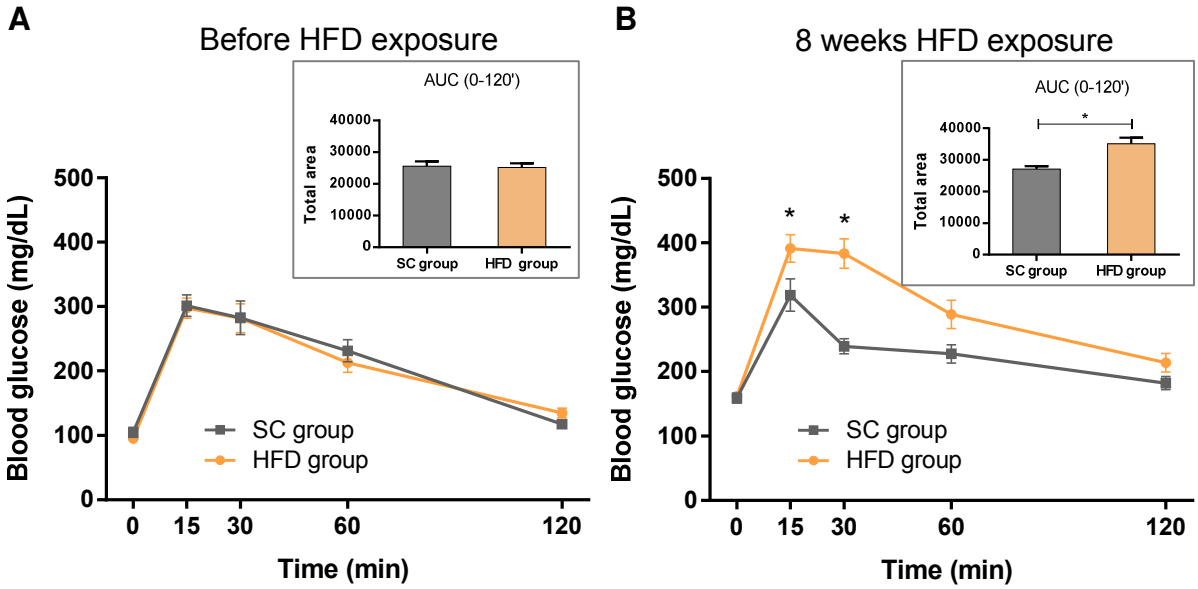


Figure 2.2. Blood glucose levels in control (SC-fed) and experimental (HFD-fed for 8 weeks) mice challenged with a i.p. bolus of glucose (2 g/kg) in the glucose tolerance test (GTT). The SC group comprised 12 animals, the HFD group comprised 12 animals. **(A)** Results from GTT at baseline (before introduction of HFD to half of the animals) where glucose was monitored at baseline (0 min) and 15, 30, 60 and 120 min after the bolus of glucose. Upper panel shows area under the curve (AUC) for 0-120 min. **(B)** Results from GTT after HFD exposure; glucose was measured at baseline (0 min) and 15, 30, 60 and 120 min after i.p. glucose injection. Panel shows area under the curve (AUC) for 0-120 min. Glucose levels are shown as mean ± SEM, * indicates $p < 0.05$.

Having started with similar blood glucose levels (**Figure 2.2A**), HFD-exposed mice showed significantly higher levels of this metabolite (**Figure 2.2B**) when tested 15 min and 30 min ($p < 0.05$) after administration of a bolus i.p. dose of glucose than did the SC-fed (control) mice (Time effect: $F_{4,110} = 44.09$, $p < 0.0001$; treatment effect: $F_{1,110} = 33.61$, $p < 0.0001$; treatment x time interaction: $F_{4,110} = 4.899$, $p = 0.0011$). Additionally, a significant difference ($p < 0.05$) was revealed by the area under the curve (AUC, 0-120 min) analysis (panel on **Figure 2.2B**).

Morphochemistry-based detection of PPARγ mRNA and protein in brain

In situ hybridization histochemistry (ISHH) was used to examine expression levels of PPARγ mRNA in brain areas concerned with the regulation of metabolism and cognitive behaviour, functions in which PPARγ has been implicated or may be expected.

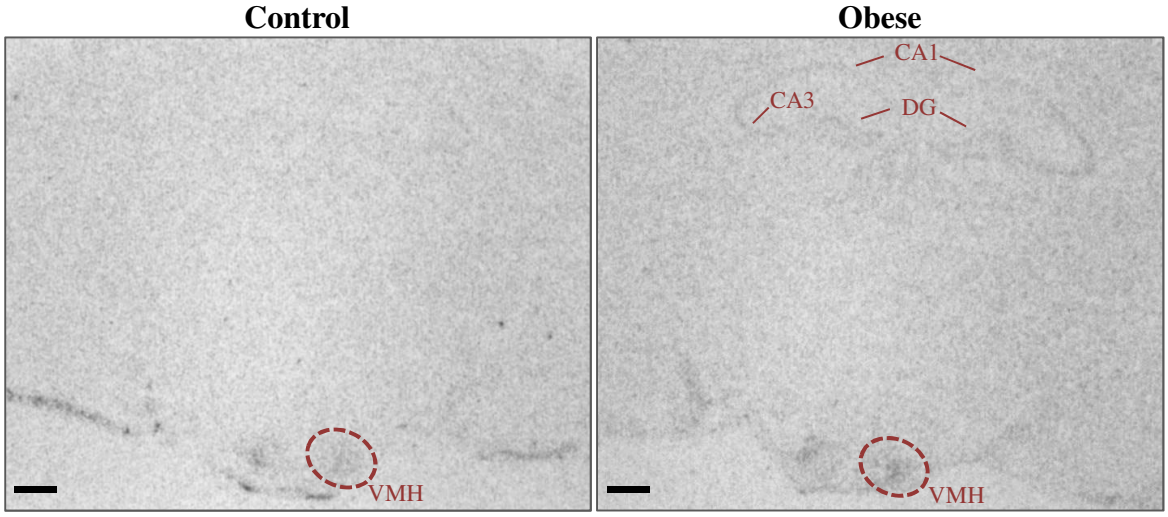


Figure 2.3. Example of PPARγ mRNA expression in the mouse brain, detected by ISHH. Coronal brain sections (Bregma - 1.82) obtained from mice receiving either standard chow (SC, controls*; left hand panel*) or rendered overweight by a high fat diet (HFD) for 8 weeks (*right hand panel*). PPARγ mRNA signal is weakly detectable in the ventromedial hypothalamus (VMH) of control animals and slightly stronger in that of HFD-fed animals. Note that whereas PPARγ mRNA is not seen in the hippocampus of control mice, weak signal is visualized in the CA1 (*cornu ammonis* area 1), CA3 (*cornu ammonis* area 3) and DG (dentate gyrus) of mice held on HFD for 8 weeks. Scale bars: 500 µm.

Whereas PPARγ mRNA was barely detectable in brain sections from control (SC-fed) mice (**Figure 2.3**, *left hand panel*), low, but consistent, transcript signal was detectable in the hippocampus and ventromedial hypothalamus (VMH) of mice rendered overweight and glucose intolerant by exposure to a chronic HFD (**Figure 2.3,** *right hand panel*). The PPARγ mRNA signals obtained were not sufficiently strong to allow semi-quantitative analysis.

As shown in **Figure 2.4**, relatively high PPARγ protein expression was observed by immunohistochemistry in fresh-frozen (poor preservation of morphology) brains from control (SC-fed) mice, with immunoreactivity being seen in both the cell nucleus and cytoplasm. Nevertheless, the wide and diffuse signals detected give reason for concern about antibody specificity, especially because tests with antibodies from different sources also gave results that indicated lack of site-specificity or no signal at all.

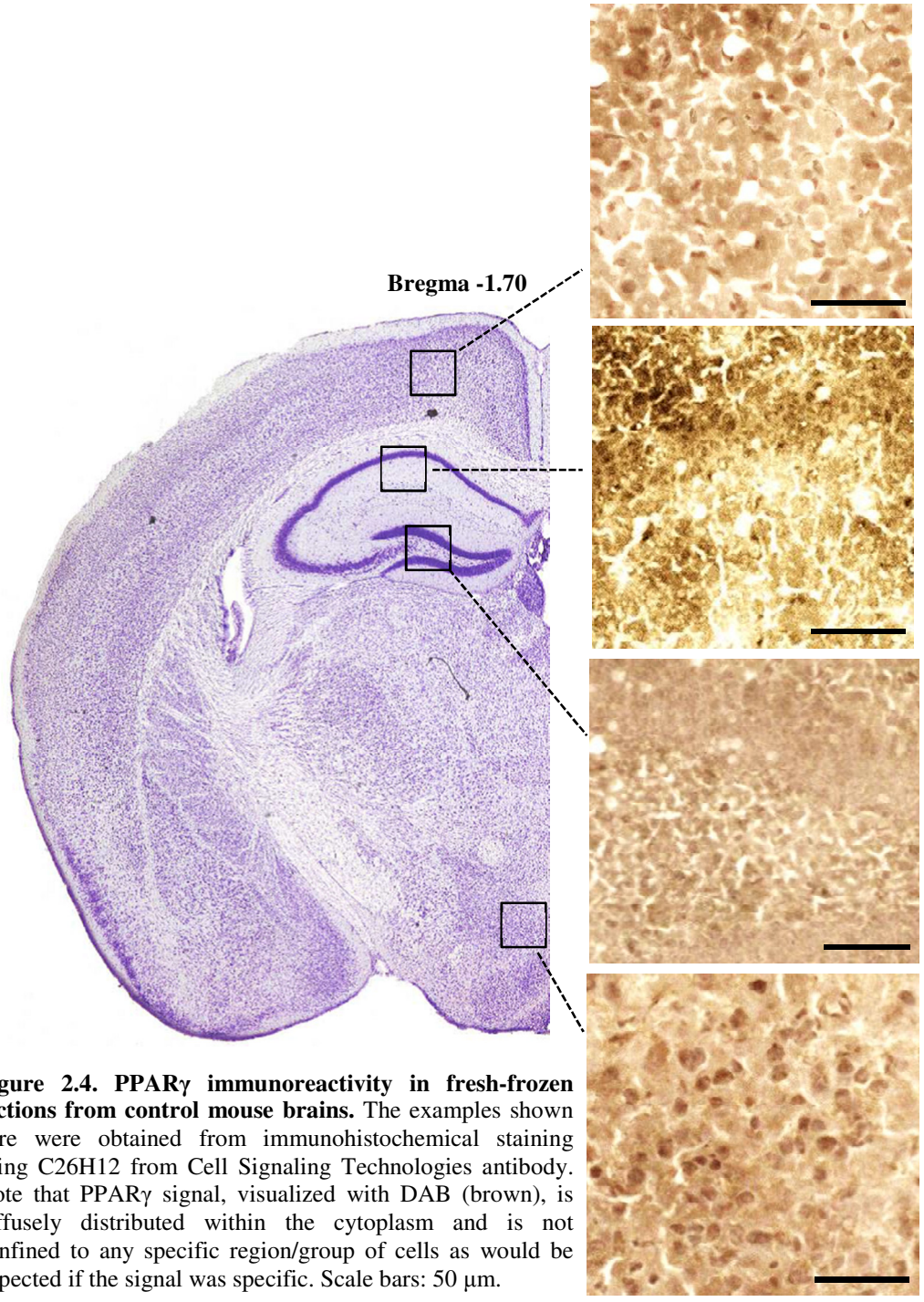


Figure 2.4. PPARγ immunoreactivity in fresh-frozen sections from control mouse brains. The examples shown here were obtained from immunohistochemical staining using C26H12 from Cell Signaling Technologies antibody. Note that PPARγ signal, visualized with DAB (brown), is diffusely distributed within the cytoplasm and is not confined to any specific region/group of cells as would be expected if the signal was specific. Scale bars: 50 μm.

Given the poor quality of fresh-frozen preparations and the possibility that this method of tissue preservation did not properly preserve PPARγ immunoreactivity, tests were subsequently conducted on brains from PFA-perfused mice (**Figure 2.5,** *left*). While PFA perfusion somewhat improved the quality and intensity of immunoreactive signal (**Figure**

2.5, *left*), the results obtained were still of insufficient quality to conclude about specificity of the antibody or the precise localization of signal.

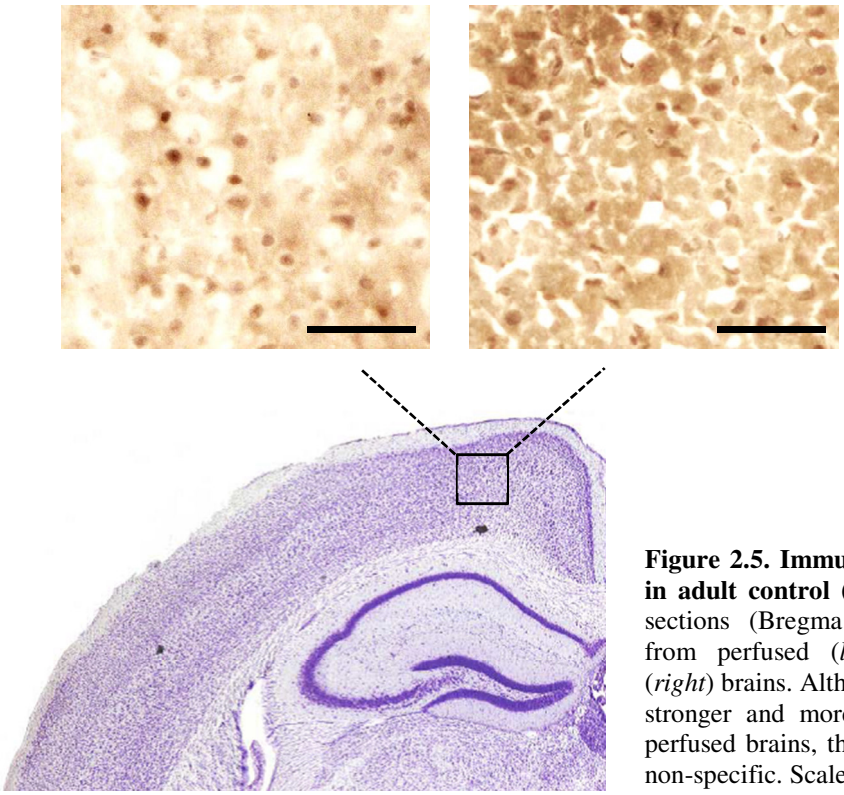


Figure 2.5. Immunodetection of PPARγ in adult control (SC-fed) mice. Coronal sections (Bregma -1.70) were obtained from perfused (*left*) and fresh frozen (*right*) brains. Although signal was slightly stronger and more discrete in the PFA-perfused brains, the results are considered non-specific. Scale bars: 50 µm.

Immunoblotting approach to detect PPARγ protein in brain

In line with the pattern of PPARγ mRNA expression (observed using ISHH), western blot analysis showed low PPARγ protein expression in prefrontal cortex, dorsal- and ventral hippocampus (**Figure 2.6**). As shown in **Figure 2.6** and **Figure 2.7** neither stress nor pioglitazone alone had a significant effect on PPARγ protein levels in any of the brain areas examined (prefrontal cortex, dorsal and ventral hippocampus, and hypothalamus). PPARγ signal was not detectable in lysates of the hypothalamus (**Figure 2.7**).

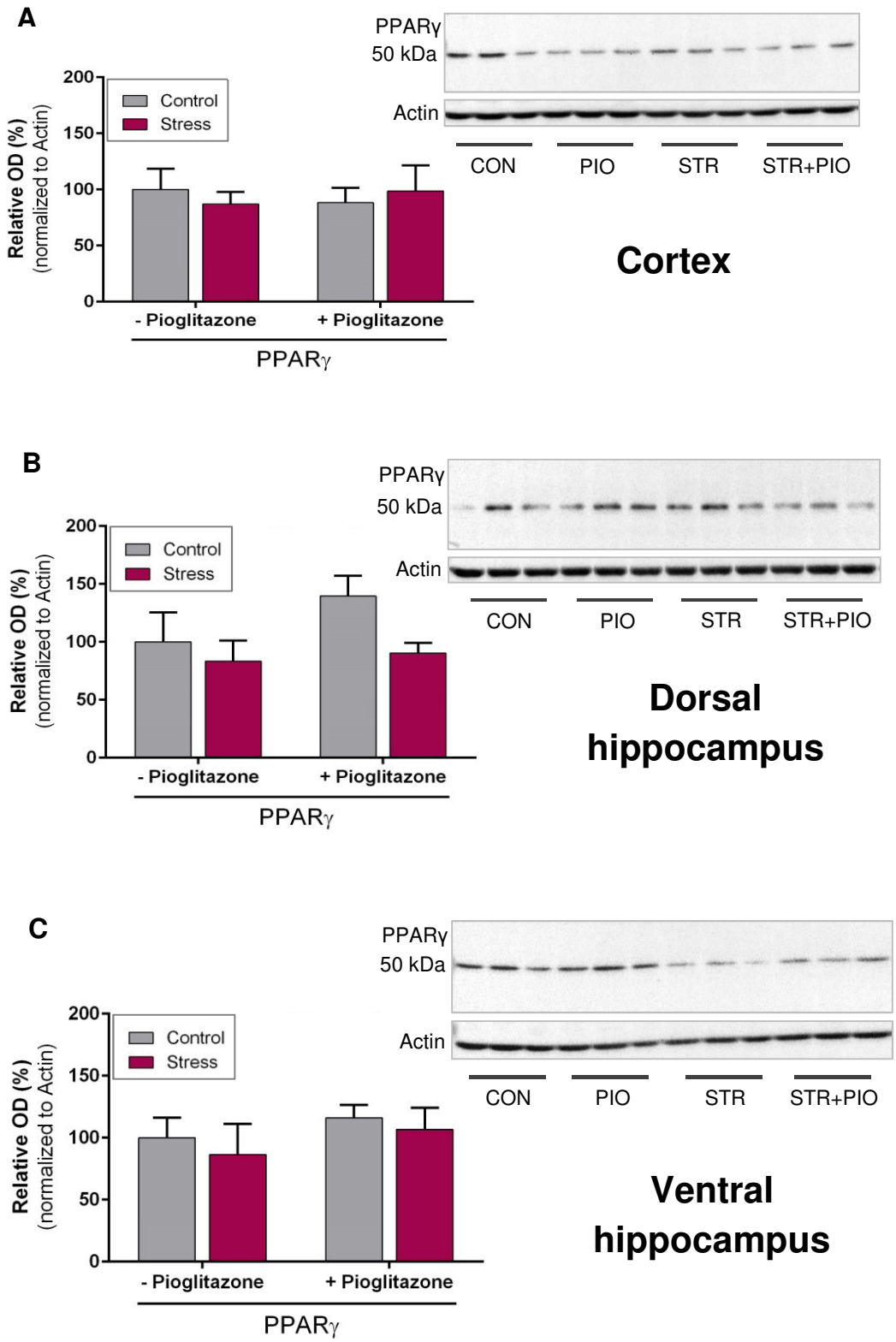


Figure 2.6. Effect of stress and pioglitazone on PPARγ protein levels. A chronic unpredictable stress (CUS) protocol was used to produce cognitive impairment. Adult mice (aged 4-5 months) received either SC or SC supplemented with pioglitazone. The western blot analysis comprised four groups: control (n=7), control+pio (n=7), stress (n=6) and stress+pio (n=7). Immunoblotting (left panel) was conducted to detect PPARγ protein (50 kDa) in **(A)** Prefrontal cortex, **(B)** Dorsal hippocampus and **(C)** Ventral hippocampus. Right panel shows the quantification of protein signal in each area, respectively. PPARγ appears to be expressed at low levels in the brain of mice. Data are presented as ± SEM, * indicates $p < 0.05$.

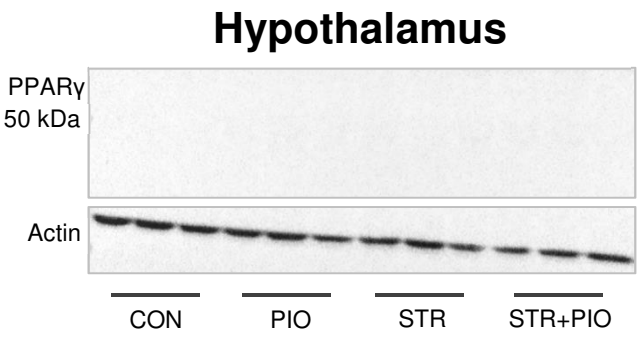


Figure 2.7. Effect of stress and pioglitazone on PPARγ protein levels in hypothalamus. A chronic unpredictable stress (CUS) protocol was used to produce cognitive impairment. Adult mice (aged 4-5 months) received either SC or SC supplemented with pioglitazone. The experiment consisted of four groups: control (n=7), control+pio (n=7), stress (n=6) and stress+pio (n=7). PPARγ protein (50 kDa) was not detectable in hypothalamus by immunoblotting.

2.4 Discussion

This work was undertaken in view of the suggested therapeutic value of PPARγ stimulation in brain disorders such as AD (Heneka and Landreth, 2007), as well as in the regulation of hypothalamic circuits that control feeding and energy metabolism (Diano *et al*., 2011; Lu *et al*., 2011; Ryan *et al*., 2011; Garretson *et al*., 2015; Liu *et al*., 2015). Since imbalances in glucose and lipid metabolism are thought to increase the risk for AD (Heneka *et al*., 2015b; Luchsinger, 2012; Merlo *et al*., 2010; Pérez and Quintanilla, 2015; Rasgon and Kenna, 2005) and PPARγ agonists act as anti-diabetic agents by increasing sensitivity to insulin (Lehmann *et al*., 1995; Hanyu *et al*., 2009; Sato *et al*., 2011; Heneka *et al*., 2015b), the question of whether PPARγ agonists ameliorate AD by acting directly in the brain or by improving peripheral metabolism is an important one. The efficacy of direct PPARγ agonist effects on neural substrates relevant to AD would depend on the presence of PPARγ in areas such as the cortex and hippocampus. As reviewed in the introduction to this chapter, the available evidence for the expression of PPARγ in the brain is poor. This study represented a further attempt to strengthen the existing knowledge base through the use of biochemical and morphochemical methods to examine whether the adult mouse brain expresses PPARγ and whether any receptors found are subject to regulation by physiological and pharmacological stimuli.

Analysis by *in situ* hybridization histochemistry (ISHH) showed that PPARγ mRNA is expressed at very low levels in the mouse brain under baseline (standard lab-holding and diet) conditions (**Figure 2.3**, *left hand panel*). However, exposure of mice to a high fat diet (HFD) that not only induced overweight (**Figure 2.1B**), but also (predictably)

glucose intolerance (**Figure 2.2B**), led to an upregulation of PPARγ mRNA levels in the hippocampus and in the ventromedial nucleus of the hypothalamus (VMH) (**Figure 2.3,** *righ hand panel*); the latter area plays an important role in feeding behaviour, directly responding to glucose and a variety of other feeding- and energy-regulatory neurotransmitters (King, 2006). However, because the PPARγ transcript signal was so low, even under the HFD regimen, quantitation and comparison between results in control and HFD-fed animals could not be made. It should also be mentioned that an attempt was made in our own previous studies to detect PPARγ mRNA in mouse brain, using quantitative polymerase chain reaction (qPCR) assays, albeit without any positive results (and therefore not reported above). It is however interesting to note that other authors observed that HFD increases PPARγ mRNA expression (and peroxisome numbers) in the hypothalamus (Diano *et al.,* 2011).

While mRNA measurements usually provide a good indication of whether a specific gene is expressed in a particular tissue, mRNA species could escape detection if they have a high turnover rate. In many cases, gene products (proteins) may be more stable than mRNA. To this end, we used the immunoblotting (Western blotting) technique to examine whether PPARγ protein is present in brain areas implicated in cognitive behaviour (cortex, hippocampus), the neural regulation of the stress response (cortex, hippocampus and hypothalamus), and the control of feeding and metabolism (hypothalamus). The results of the present study indicate that the mouse brain exhibits only low levels of PPARγ protein (indirectly confirming the low mRNA levels found). Moreover, neither stress nor pioglitazone influenced PPARγ protein expression significantly (despite a tendency for pioglitazone to upregulate PPARγ protein levels, see **Figure 2.6**).

Since the distribution of many proteins is confined to specific subsets of neurons within a given brain area, the results from immunoblotting of tissue lysates can be confounded by "dilution effects" (for the same reason, qPCR results may also be limited or misleading). Accordingly, we next sought PPARγ protein expression in discrete brain nuclei using immunohistochemistry, a method that has the potential to offer high sensitivity and spatial resolution, provided that the specimens are optimally preserved (fixed) so as to conserve both tissue/cellular integrity and antigenicity. Using fresh-frozen sections, we here observed high, but diffuse, PPARγ protein expression in the cortex (**Figure 2.4**). However, because of the poor preservation of morphology in fresh-frozen tissue, it

proved difficult to conclude about signal specificity. For example, the diffuse signal appeared to be present in both cytoplasm and nucleus although the expectation would be that, like other nuclear receptors, PPARγ would be mainly localized in the latter. Optimization of the immunostaining protocol (e.g. use of antigen-unmasking with citrate buffer or signal intensification using avidin-biotin-peroxidase complex) did not resolve this problem. Improved staining was obtained when brains from PFA-perfused animals were used, but the need to cut these brains on a cryostat (paraffin sectioning not available) also compromised morphological quality and interpretation.

In summary, the work undertaken here failed to find convincing evidence that the adult mouse brain expresses significant and regulatable amounts of PPARγ mRNA and/or protein. The PPARγ mRNA results obtained here concur to some extent with previous reports but, also differ from others (an extensive search of the literature did not reveal consistent reports of PPARγ protein expression in the brain of mice using immunohistochemical or immunoblotting methods). The reasons for the discrepancy between the various studies are hard to identify. However, we suggest that detection of PPARγ in the adult mouse brain depends on one or more of the following variables: specific line or source of mice, diet, housing conditions (e.g. temperature), tissue collection, storage and preparation for assay, specific reagents and/or protocols that have not been fully published. Age is another very likely important factor since PPARγ mRNA levels were found to decrease from embryonic days 13.5 to 15.5 (E13.5-E15.5), reaching undetectable levels by E18.5 in the CNS of rats (Braissant and Wahli, 1998). Indeed, in parallel studies in our lab (carried out by Susanne Moosecker), PPARγ mRNA and protein is detectable in cultured hippocampal neurons as well as hippocampal astrocytes and oligodendrocytes (<< neurons) derived from 4-day old mice (*unpublished).* In conclusion, progress in the understanding of how PPARγ influences brain function (with the perspective of developing and applying PPARγ target drugs) will depend on the generation of better reagents (in terms of specificity and sensitivity) and methodological approaches to detect basal PPARγ levels in the brain, as well as to monitor how this receptor is regulated after physiological, pathological and pharmacological manipulations. Until then, the mechanisms through which administered PPARγ agonists influence brain and behaviour are likely to be judged as secondary, raising issues of potential undesired outcomes, especially when such agonists must be applied chronically.

CHAPTER 3

Pioglitazone actions during stress and its ability to prevent functional decline over time

Abstract

Stress contributes to the development of various diseases and disorders, including diabetes and obesity, mood disorders and Alzheimer's disease (AD). The peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated nuclear receptor, is a key regulator of adipogenesis, lipid and glucose metabolism. PPARγ is implicated in obesity; its agonists are effective promoters of insulin sensitivity and are therefore sometimes used to treat type 2 diabetes (T2D). In addition, recent studies have implicated PPARγ in AD as well as in the regulation of stress; the latter is thought to be a likely trigger of AD. Given the association between metabolic disorders and AD, this study examined whether PPARγ activation in adult male mice might delay chronic stress-induced AD-like pathology at the behavioural and biochemical levels. Our results show that chronic unpredictable stress (CUS) produces weight loss and, glucocorticoid (GC) hypersecretion, while impairing glucose tolerance and insulin sensitivity. Pioglitazone, a PPARγ agonist, reverses the metabolic effects of CUS but fails to reverse CUS-induced hyperlocomotion, and increases body weight and white adipose tissue mass. Pioglitazone treatment may have contributed to reduce motivation for a food reward and to a deficit in appetitive learning capacity. Confirming previous findings, it was found that CUS increases the levels of tau (an AD-related protein) and of aberrantly phosphorylated forms of the protein in specific brain regions; these changes were not influenced by pioglitazone. In summary, the herein presented results strongly suggest a role, for PPARγ in linking stress, metabolism and cognitive function; however, the mechanisms underlying this interaction remain enigmatic. Given the strong cause-effect relationships between stress, metabolism and cognitive dysfunction, further studies are warranted as they may provide clues for therapeutic improvements.

3.1 Introduction

Continuous exposure to increased glucocorticoid (GC; mainly cortisol in humans and corticosterone in rodents) levels are associated with health problems, such as hypertension and metabolic disorders (e.g. obesity, type 2 diabetes). The relationship between stress and type 2 diabetes (T2D, also called diabetes mellitus) is bidirectional, with hypercortisolemia causing insulin resistance and *vice versa* (Rasgon and Kenna, 2005; Zardooz *et al*., 2006; Depke *et al*., 2008; Rostamkhani *et al*., 2012; Ghalami *et al*., 2013; Li *et al*., 2013). Interestingly, some patients receiving GC therapy may also develop the so-called steroid-induced diabetes (Hwang and Weiss, 2014). On the other hand, stress is considered to be a primary trigger of neuropsychiatric conditions such as mood disorders and cognitive decline; the latter are thought to result from synaptic loss and neuronal atrophy, (Sapolsky, 2000; Sotiropoulos *et al*., 2008a,b; Catania *et al*., 2009; Chrousos, 2009; Sotiropoulos *et al*., 2011; Lopes *et al*., 2016; Sotiropoulos and Sousa, 2016). Of particular relevance to this thesis are reports linking T2D with a higher risk for developing AD (Merlo *et al*., 2010; Luchsinger, 2012; Pérez and Quintanilla, 2015; Heneka *et al*., 2015b), with hypercortisolemia being a common event in patients with AD (Hartmann *et al*., 1997; Weiner *et al*., 1997; Csernansky *et al*., 2006; Elgh *et al*., 2006; Sotiropoulos *et al*., 2008b). Our group previously demonstrated that exposure to chronic stress or exogenous GCs can lead to the production of AD-like pathobiochemistry and behaviour (impaired memory) in rats by increasing the misprocessing of amyloid precursor protein (APP) into amyloidogenic peptides (e.g. amyloid beta, Aβ), amyloid deposition and the inappropriate hyperphosphorylation of tau protein (Sotiropoulos *et al*., 2008a; Catania *et al*., 2009; Sotiropoulos *et al*., 2011). Work by other groups in which transgenic mouse models of AD were used made similar observations (Green *et al*., 2006; Jeong *et al*., 2006).

PPARγ is a ligand-activated nuclear receptor, which is critically involved in adipocyte differentiation, fatty acid storage and glucose metabolism (Lehrke and Lazar, 2005; Cho *et al*., 2008; Tontonoz and Spiegelman, 2008; Wahli and Michalik, 2012). Some studies suggest that PPARγ signaling may contribute to the regulation of physiological response to stress. The evidence leading to this view is mainly correlational, based on the finding that cerebral cortical PPARγ expression is increased after exposure of rats to acute restraint stress (García-Bueno *et al*., 2005a; García-Bueno *et al*., 2008a), and that

inhibition of GC-synthesis or glucocorticoid receptor (GR) antagonism prevents the stress-induced up-regulation of PPARγ expression and activity in the brain (García-Bueno *et al*., 2008a). On the other hand, although PPARγ agonist treatment reduces corticosterone (CORT) levels after stress, it does not affect the secretion of adrenocorticotropin (ACTH), the pituitary hormone that stimulates GC secretion (Ryan *et al*., 2012). Additionally, treatment with PPARγ agonists prevents the stress-induced increases in the production of pro-inflammatory peptides (TNFα, NOS-2, COX-2) (García-Bueno *et al*., 2005a,b; García-Bueno *et al*., 2008a,b). Interestingly, two studies reported that thiazolidinediones (TZDs), a class of drugs to which most PPARγ agonists belong, may also be partial GR agonists (Ialenti *et al*., 2005 and Matthews *et al*., 2009).

Numerous studies have reported that PPARγ agonists act in transgenic mouse models of AD, carrying human APP mutations that induce cerebral amyloid plaques (producing plaque pathology), to improve learning and memory deficits (Pedersen *et al*., 2006; Escribano *et al*., 2009; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Rodriguez-Rivera *et al*., 2011; Denner *et al*., 2012; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Jahrling *et al*., 2014; Chen *et al*., 2015; Yu *et al*., 2015); these behavioural effects are accompanied by reduced Aβ and tau deposits in the brain (Pedersen *et al*., 2006; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012). Importantly, TZDs were also found to retard memory decline in patients with mild-to-moderate AD (Watson *et al*., 2005; Risner *et al*., 2006) and in diabetic patients with mild AD (Hanyu *et al*., 2009; Sato *et al*., 2011). Further, T2D patients that received TZDs, rather than metformin, were found to have decreased risk for dementia (Heneka *et al*., 2015b).

This study was undertaken in light of the potential importance of PPARγ in the regulation of stress and metabolism and their impact on cognitive behaviour relevant to AD. Specifically, we examined whether the deleterious effects of chronic unpredictable stress (CUS) in mice are reversible with pioglitazone (PIO), a PPARγ agonist, and/or whether PIO can delay the onset of cognitive deficits.

3.2 Materials and Methods

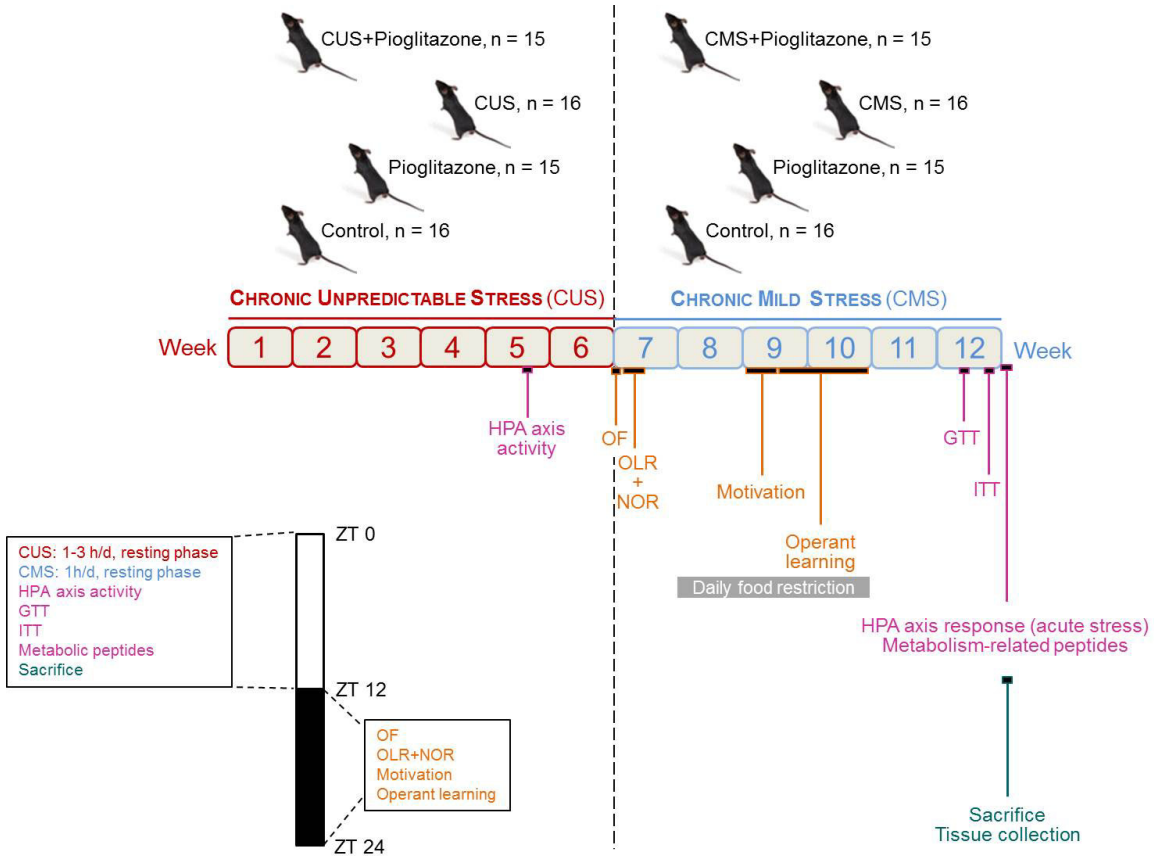
Animals and tissues

Male mice (C57/BL6 strain, Martinsried, Germany), aged 4-5 months (n=62) were used in compliance with the European Union Council's Directive (2010/63/EU) and local regulations. Animals were housed 4 per cage under standard laboratory conditions [temperature 22 °C; relative humidity 50 ± 10%; 12h light/dark cycle (lights on at 7:00)], *ad libitum* access to food (#1324 laboratory diet; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and water, unless otherwise mentioned. Behavioural tests were performed at the end of 6 weeks of exposure to chronic unpredictable stress (CUS, see below) during animal's resting period (diurnal phase). Baseline individual body weights were monitored before CUS exposure and at weekly intervals thereafter. At the end of the experiment, animals were sacrificed by rapid cervical dislocation and decapitation. Their brains and visceral fat depots were carefully removed. Brains were rapidly dissected on ice to separate the prefrontal cortex, hypothalamus, dorsal and ventral hippocampus, which were then snap-frozen in isopentane (2-methylbutane; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) on dry ice, before storage at -80° C for subsequent biochemical analyses. Adipose tissue was weighed and stored at -20° C.

Chronic unpredictable stress (CUS)

After 2 weeks of habituation, mice were divided into two groups: stressed and unstressed (controls). Stressed animals were exposed to a chronic unpredictable stress (CUS) paradigm for 6 weeks, comprising exposures (1-3 h) to one of the following stimuli, applied in a random order and at unpredictable times of the day, using a slight modification of a published protocol (Catania *et al*., 2009; Sotiropoulos *et al*., 2011): 1) overcrowding (4 mice in a space measuring 12.75 cm x 9.75 cm x 6.9 cm), 2) placement of cages (4 mice/cage) on a vibrating plate, 3) strong, air puffs delivered with a hairdryer and 4) restraint stress (individual mice placed in a 50 ml conical tube with an breathing hole at front and a hole through which the tail was extended). Control animals were held undisturbed and handled weekly. After the 6 weeks of CUS, animals were exposed to the same stressful stimuli as mentioned before, but over a shorter duration (maximum 60 min) and at the same time every day (also referred to as chronic mild stress, CMS); this

was aimed at maintaining some of the damaging effects of CUS which are known to diminish over time (Sousa *et al.*, 2000).



Scheme 3.1. Experimental design. A 6-week chronic unpredictable stress (CUS) protocol was used in a group of animals to produce metabolic and cognitive dysfunction. Control and stressed animals were further divided into subgroups receiving pioglitazone (3.3 mg/kg BW) in their diet. Behavioural testing followed CUS while animals were exposed to chronic mild stress (CMS) for a further 6 weeks. Animals received stressors during the light (resting) phase and were tested during the daily period of darkness (active) phase. Blood samples were collected during CUS to determine HPA axis activity. During the last week of the experiment, glucose tolerance (GTT) and insulin tolerance (ITT) tests were conducted. Just before sacrifice, animals were exposed to an acute stressor and blood samples were collected 30 minutes later. Tissues were collected after sacrifice and stored until further analysis (see main text).

Pioglitazone treatment

Half of the animals [control (PIO) and stress (STR+PIO), n = 15 per group] were given pioglitazone for a total of 12 weeks (see **Scheme 3.1**). For this, pioglitazone (Actos™ Takeda Pharma A/S, Denmark) was given in the diet (#1324 laboratory diet, Altromin, Lage, Germany) at a concentration of 20 mg/kg (estimated dosage of 3.3 mg/kg BW, assuming food consumption of 5 g of chow per animal/day). The other half of the animals [control (CON) and stress (STR), n = 16 per group] were maintained on standard diet

(#1324 laboratory diet, Altromin, Lage, Germany). Food and water were available *ad* libitum.

Behavioural tests

Open field test **-** The open field test (OF) was performed to examine exploratory behaviour and general locomotor activity. The apparatus consisted of a plexiglass arena measuring 33 x 33 x 30 cm (white base; grey walls). The test was conducted under low illumination (50 lux) to avoid anxiety. Each mouse was placed in the arena and allowed to explore it for 5 min. Activity was recorded by a video camera and analysis was conducted using ANY-maze software (Stoelting, Wood Dale, IL, USA). Total distance travelled (m), immobility time (s) and number of line crossings were analyzed.

Novel object location and novel object recognition tests – The protocols followed were slightly modified after Barker and Warburton (2011) and Leger *et al*. (2013). Animals were habituated to the OF arena (see above) for 10-15 minutes daily for 2 days before testing. Testing was done under dim (15 lux) light and consisted of a sample phase and two recognition (object location recognition and novel object recognition) task phases, the delay between the sample phase and object location recognition test being 1 h, and between the sample phase and the novel object recognition test being 24 h. For the recognition tests, mice had to explore the objects for at least 5 sec; animals that explored <5 sec were excluded from the statistical analysis. Specifically, for the

- ✓ ***sample or familiarization phase,*** two identical objects were placed equidistantly near one wall of the arena. Animals were placed facing the opposite wall and allowed to explore the arena (including objects) for 10 min. Activity and individual exploration patterns were recorded by a video camera. Exploration was defined as "directing the nose toward the object at a distance less than 2 cm" (Leger *et al*., 2013. Nature protocols) and scored manually.
- ✓ ***object location recognition task (OLR),*** after a 1 h delay (after the sampling phase), one of the objects was moved to a new location and animals were allowed to explore the objects, for 10 min (as above); relocation of the object was counterbalanced between groups. Exploration time of the objects was measured and results were used to obtain a discrimination ratio, computed as follows:

$$Discrimination\ ratio\ (\%) = \frac{t\ (novel\ side) - t\ (familiar\ side)}{Total\ time} * 100$$

- ✓ ***novel object recognition task (NOR),*** 24 h after the sample phase, mice were exposed to objects located in the same positions as in the sample phase, but one of the objects was a novel one; novel and familiar objects and their positions were counterbalanced between animals. Time exploring each object was measured and discrimination ratios were calculated as follows:

$$Discrimination\ ratio\ (\%) = \frac{t\ (novel\ object) - t\ (familiar\ object)}{Total\ time} * 100$$

Motivation

Animals were food restricted over **7** days to reach 10-15% loss of initial BW; food restriction was continued to maintain this (reduced) BW during the period of behavioural testing.

Motivation was tested as previously described (Harb *et al*., 2014; also see Horner *et al*., 2013). Briefly, the test was carried out over 3 consecutive daily sessions in mouse touchscreen chambers. During each session, mice received 15 liquid food reward (15 µl condensed milk with 14% sugar) presentations delivered at a variable interval of 10-40 sec. Reward retrieval latency, time to retrieve reward, food tray entries/min and activity (beam breaks/min) were analyzed to evaluate motivation.

Operant (instrumental) conditioning

Operant conditioning was performed in mouse touchscreen chambers as described by Harb *et al*. (2014) (cf. Horner *et al*. 2013). Briefly, food-restricted (as described above) mice were presented with 20 light stimuli at the centre of the touchscreen at a variable interval of 10-40 sec. Animals received a liquid food reward (15 µl condensed milk with 14% sugar) each time they nose-poked the screen. The test consisted of 1 daily session which lasted a maximum of 45 min or until criterion (finish 20 trials in less than 20 min on at least 3 consecutive days) was reached. Animals that did not reach criterion by the 8th day of testing were excluded from statistical analysis. Instrumental learning was evaluated on the basis of the following measures: 1) trials completed/session, 2) time to complete session, 3) stimulus touches/min, and 4) activity (beam breaks/min).

Intraperitoneal (i.p.) glucose tolerance test (GTT)

Following a fast of 16 h, a small (approximately 10 µl) tail vein blood sample was withdrawn from each mouse. Basal blood glucose concentrations were measured using a glucometer (OneTouch® Vita®, LifeScan; Johnson and Johnson Medical, Neuss, Germany), after loading blood samples (capillary action) onto calibrated test-strips provided by the manufacturer. Mice received an i.p. injection of 20% glucose (Sigma) at a dose of 2 g/kg, in a volume of 0.01ml/g body weight (BW). Thereafter, tail blood samples were withdrawn (15, 30, 60 and 120 min) for determination of glucose levels. Mice were returned to their home cages between each sampling.

Intraperitoneal (i.p.) insulin tolerance test (ITT)

Mice were fasted for 6 h before providing a tail blood sample for determination of basal blood glucose levels (as above). Subsequently, they received an i.p. injection of 0.75U/kg insulin (Humalog; Eli Lilly and Company, Bad Homburg, Germany) in a volume of 7.5 µl/g BW, and blood glucose titres were measured at 15, 30, 60 and 120 min post-exogenous insulin. Animals were allowed to rest in their home-cage between each sampling.

Metabolism-related peptide assays

Blood serum obtained at the time of sacrifice was assayed for insulin and leptin using a Luminex®-based technology Milliplex MAP kit (#MMHMAG-44K-05 & #MCYTOMAG-70K-06 for mouse; Merck Chemicals, Am Kronberger, Schwalbach, Germany), according to manufacturer's instructions. Analyte's concentrations were detected using a Bio-Plex® system (Bio-Rad Laboratories GmbH, Munich, Bayern, Germany). Readings that were undetectable were assigned the value of the respective minimum detectable concentration stated by the Milliplex kit manufacturer (insulin: 14 pg/ml; leptin: 19 pg/ml).

Assessment of hypothalamic-pituitary-adrenal (HPA) axis activity

Tail vein blood samples were obtained during the fifth week of CUS to assess the efficacy of the applied stress paradigm by measuring serum concentrations of corticosterone using a sensitive radioimmunoassay (RIA) (RIA-1364; DRG Instruments GmbH, Marburg, Germany) with a lower limit of detection of 7.7 ng/ml.

The dynamic response to an acute stressor (abrupt shaking, 2 min) was analyzed on the last day of the study. The stressor was applied 30 min before sacrifice when trunk blood was obtained to measure serum corticosterone levels as described above.

Immunoblotting

Frozen brain areas of interest (prefrontal cortex, hippocampus, hypothalamus) were dounce- homogenized in homogenization buffer[10] and centrifuged (14.000 *g*, 4 °C, 15 min) to obtain protein-containing lysates that were stored at -80 °C until used. Protein concentrations in the thawed (on ice) lysates were determined by Lowry's method (Lowry *et al*., 1951), using a Synergy-HT plate reader (BioTek Instruments, Winooski, VT, USA) at an absorbance of 750 nm. Lysates were subsequently subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins according to their size. For this, samples (40 µg protein) were mixed with 6x Laemmli buffer[11], diluted in dH_2O to a volume of 20 µl and heat denatured (95 ºC, 5 min) before loading on to 10% acrylamide gels, along with a pre-stained protein molecular weight marker (Thermo Fischer, Braunschweig, Germany). Electrophoresis was performed (75 V, 1.5-2 h) in an electrophoresis chamber (BioRad Laboratories) filled with 1xSDS-running buffer (10xSDS[12] diluted 1:10 in dH_2O). Separated proteins were semi-dry transferred onto 0.2 µm nitrocellulose membranes (Trans-Blot Turbo Mini Nitrocellulose Transfer pack; BioRad) using the Biorad Turbo Transfer System (2.5A, 25 V, 10 min). Membranes were stained with Ponceau-S Solution (Sigma-Aldrich) to validate the quality of protein transfer, washed in TBS-T (10xTBS-T[13] diluted 1:10 in dH_2O) and incubated (1 h, RT, with shaking) in blocking solution (5% non-fat milk powder in TBS-T) before incubation (overnight, 4°C, with shaking) with primary antibodies (**Table 3.1**); except for the anti-PPARγ which was diluted in 5% bovine serum albumin in TBS-T, all primary antibodies were diluted in blocking solution. Following incubation, membranes were washed extensively with TBS-T before incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (**Table 3.2**); secondary antisera were diluted in

[10] **Homogenization buffer**: 100mM Tris (Sigma-Aldrich) pH 8.0, 1mM EDTA, 250 mM NaCl (ROTH), 5 mM $MgCl_2$ (Sigma-Aldrich), 10% Glycerol (Sigma-Aldrich), 1% Nonidet P-40 (Fluka Chemie), 20 µl of Roche 50xProteinase inhibitor cocktail tablet (diluted in 10 ml dH2O), 10 µl of each Phosphatase inhibitor cocktail (2+3, Sigma-Aldrich).

[11] **6xLaemmli Buffer**: 3.5 ml 4xStacking gel buffer*, 1.5 ml Glycerol (Sigma-Aldrich), 0.465 g DTT, 0.5 g SDS (Sigma-Aldrich), 0.6 mg Bromophenol blue (Sigma-Aldrich), dissolved in 5 ml dH_2O (storage at -20°C).
*_**Stacking gel buffer**_: 6.05 g Tris pH 6.8 and 0.4 g SDS dissolved in 100 ml dH_2O.

[12] **10xSDS:** 30 g Tris (Sigma-Aldrich), 144 g glycine (ROTH), 1 g SDS in a volume of 1 L dH_2O

[13] **10xTris-buffered saline-Tween (10xTBS-T):** 1Volume 1M Tris pH 7.5-8.0, 1Volume 3M NaCl, 1% Tween-20 (ROTH), up to 2 L dH_2O

blocking solution. Finally, membranes were extensively washed in TBS-T, placed in Lumi-Light Western Blotting Substrate (Roche, Mannheim, Germany) before visualization of proteins on a chemiluminescence detection system (ChemiDoc MP Imaging System; BioRad). Protein signals were semi-quantified with the help of ImageLab 5.1 Software (BioRad).

Table 3.1. Primary antibodies used in immunobloting studies.

Antibody	Final Dilution	Supplier
Rabbit monoclonal anti-Tau (phospho S202)	1:5000	Abcam; ab108387
Rabbit monoclonal anti-Tau (phospho S396)	1:10000	Abcam; ab109390
Rabbit monoclonal anti-Tau (phospho T231)	1:1000	Abcam; ab151559
Mouse monoclonal anti-Tau (TAU-5)	1:1000	Abcam; ab80579
Mouse monoclonal anti-Actin	1:5000	Chemicon; MAB1501R

Table 3.2. Secondary antibodies used in immunobloting analysis

Antibody	Final Dilution	Supplier
Goat anti-rabbit polyclonal antibody (H+L) HRP-conjugated	1:5000	Thermo Fischer; 31460
Sheep anti-mouse polyclonal antibody HRP-conjugated	1:10000	Amersham; NA931

Statistical analysis

Data were analyzed for statistical differences using Prism 6 software (GraphPad, San Diego, CA). After testing for gaussian (normal) distribution of the data set, further analysis included two-factor analysis of variance (2-ANOVA), followed by Tukey's or Bonferroni multiple comparison (*post hoc*) tests, as appropriate. In some cases, when more than two parameters were examined, multivariate analysis of variance (MANOVA) was performed, followed by LSD modified Bonferroni (*post hoc*), using the SPSS Statistical Package (Chicago, IL, USA); these latter analyses were kindly performed by Dr. Alexander Yassouridis (Munich). In all cases, a value of $p < 0.05$ was considered significant. All numerical data are depicted as mean ± standard error mean (SEM).

3.3 Results

Efficacy of CUS paradigm and dynamic response of HPA axis to acute stress

Diurnal (**Figure 3.1A**) and nocturnal (**Figure 3.1B**) serum levels of corticosterone (CORT) were significantly elevated ($p < 0.05$) in CUS-treated (STR) *vs.* control mice (CON), attesting to the efficacy of the chronic unpredictable stress (CUS) paradigm (5 weeks after its start).

Dietary pioglitazone supplementation (3.3. mg/kg BW) accentuated the effects of stress ($p < 0.05$) (**Figure 3.1 A** and **B**). Interestingly, stress reversed the pioglitazone-induced increase in the night:day ratio of corticosterone (CORT) in control (non-STR) mice (**Figure 3.1C**). There was a significant overall treatment effect ($F_{9,134} = 18.20$, $p < 0.0001$) in all parameters analyzed (diurnal, nocturnal, night:day ratio).

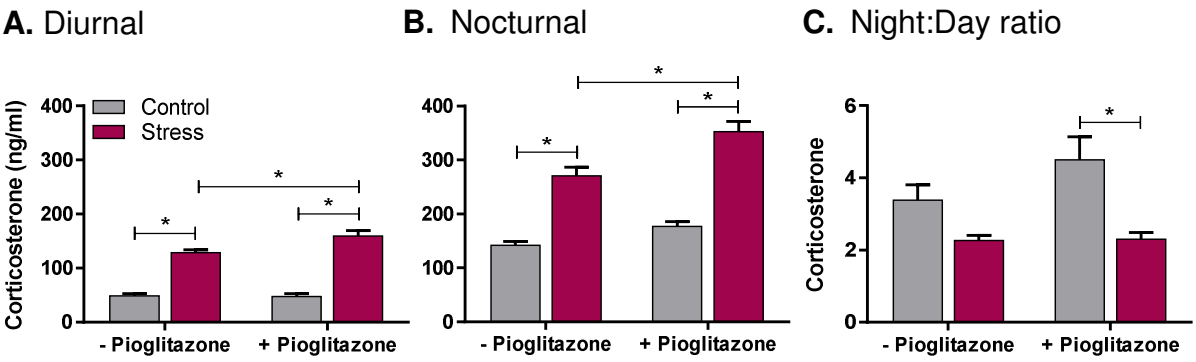


Figure 3.1. Endocrine response to CUS in male mice. Blood samples were collected during the fifth week of CUS during **(A)** the day and **(B)** night. **(C)** The nocturnal/diurnal ratio of serum corticosterone levels. The control and the stress groups comprised 16 animals each. The pioglitazone groups (control + stress) consisted of 15 animals each. Corticosterone levels are shown as mean ± SEM, * indicates $p < 0.05$.

The response to an acute stressor applied 30 min before sacrifice was measured on the last day of the experiment. While exposure of control (non-STR) mice to PIO resulted in significantly ($p < 0.05$) increased basal CORT levels (**Figure 3.2A**), as shown in **Figure 3.2B**, PIO significantly attenuated the response of non-STR animals to the acute stressor ($p < 0.05$). Similar results were observed in STR group of animals, with stress decreasing ($p < 0.05$) the response to acute stress (**Figure 3.2B**).

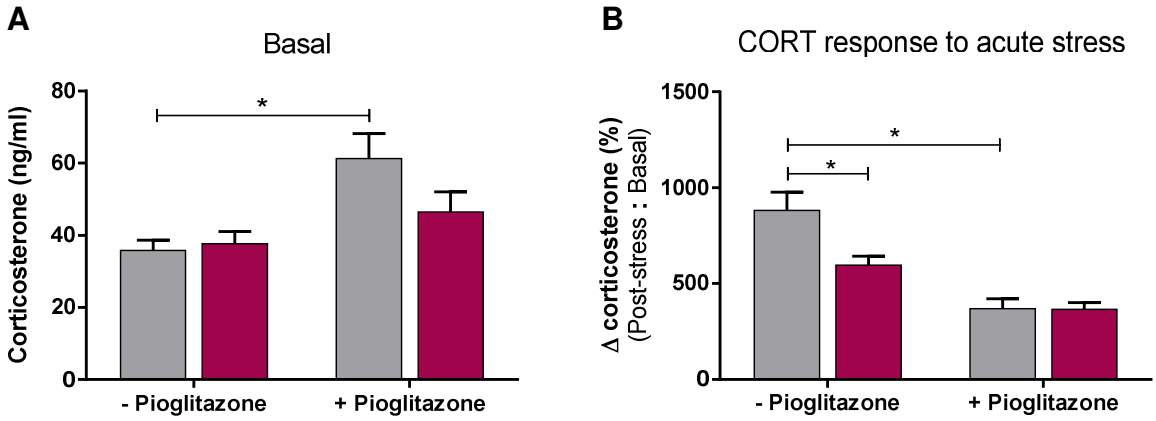


Figure 3.2. HPA-axis activity after acute stress. (A) Basal CORT levels**, (B)** CORT response to acute stress evaluated in blood of animals with 30 minutes delay. Basal CORT levels were measured before acute stress. The control and the stress groups comprised 16 animals each. The pioglitazone groups (control + stress) consisted of 15 animals each. Data are presented as mean ± SEM, * indicates $p < 0.05$.

Taken together, pioglitazone accentuated the CUS-induced increase in CORT levels, while increasing the night:day ratio. STR group displayed a lower CORT response to acute stress. Although, basal CORT levels were increased in PIO animals after 12 weeks of stress (CUS+CMS), PIO attenuated the CORT response to an acute stressor in non-STR mice.

Differential metabolic profiles in mice exposed to stress ± pioglitazone

Pioglitazone increased body weight (BW) in CON mice (**Figure 3.3A**) and prevented the BW-reducing effects of stress. These data are also presented in terms of absolute BW change in grams (**Figure 3.3B**); pioglitazone reversed the stress-induced body weight loss ($p < 0.05$) and induced a significant gain of body weight in CON+PIO mice (**Figure 3.3B**).

The changes in BW were however, not reflected in measures of white adipose tissue (WAT) mass when STR and CON were compared (**Figure 3.3C**); the small but insignificant increase in WAT mass in STR mice (despite reduced BW) suggests that STR did not entirely deplete the WAT depot, and that loss of lean tissue mass contributed to the overall loss in BW. On the other hand, PIO significantly increased WAT mass in non-STR mice, and as expected, STR counteracted this effect (**Figure 3.3C**). A complex interaction between treatment (STR/PIO), BW and WAT regulation seems to exist because, when adjusted for BW at time of sacrifice, the significant differences observed

when comparing absolute WAT mass between groups was only found in the WAT/BW of PIO-treated mice (**Figure 3.3D**).

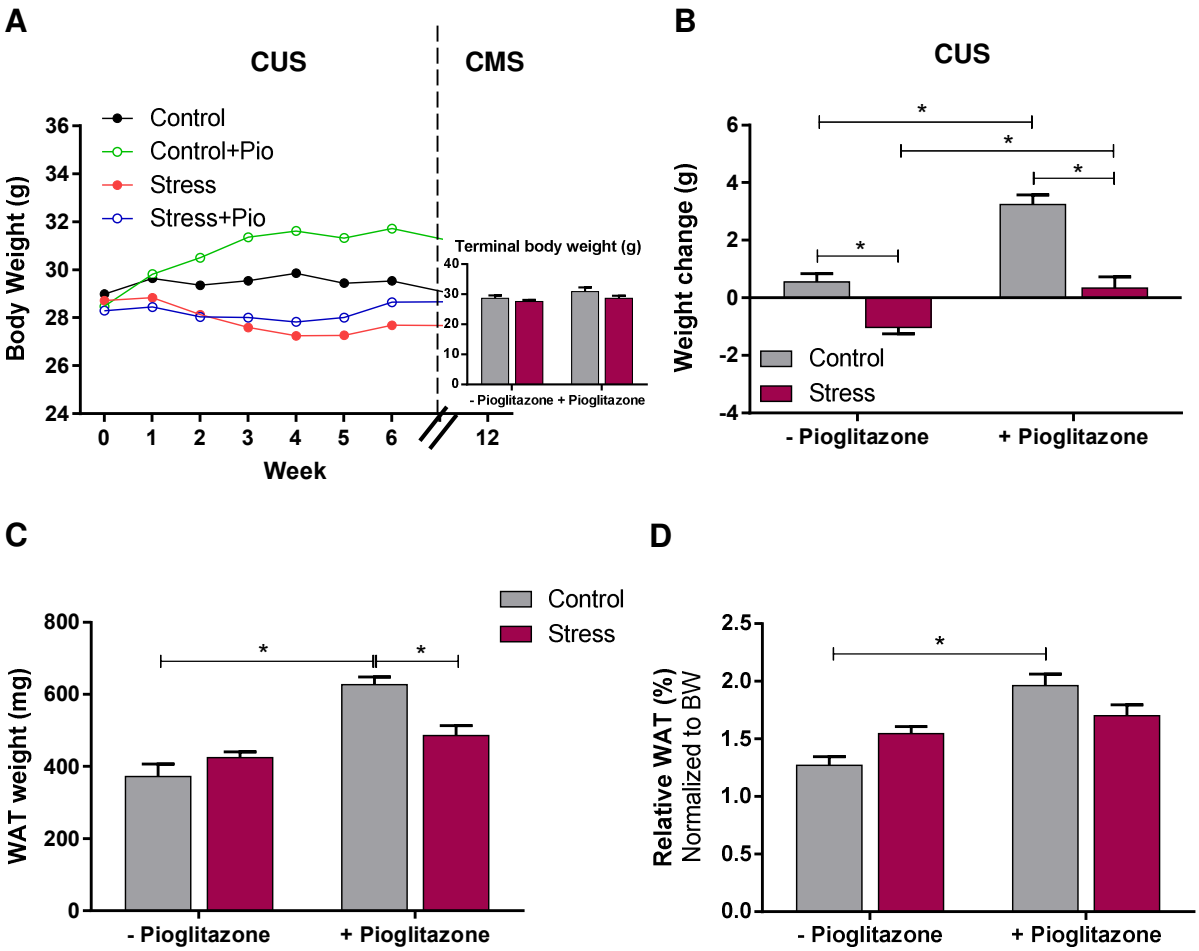


Figure 3.3. Body weight (BW) and white adipose tissue (WAT) mass in mice exposed to stress with/out oral pioglitazone treatment. (A) Body weight was monitored weekly during CUS and **(B)** body weight change was analyzed in CON (n=16), STR (n=16), PIO (n=15) and STR+PIO (n=15) groups. Inset in (A) is showing the terminal body weight of mice. **(C)** White adipose tissue (WAT) was collected and weighed on the day of sacrifice (CON, n = 10; STR, n = 13; PIO, n = 11; STR+PIO, n = 12). **(D)** WAT mass normalized to terminal body weights. Data are shown as mean ± SEM, * indicates $p < 0.05$.

Taken together, our results show that pioglitazone reverses the stress-induced BW loss and significantly increases BW and WAT mass in controls.

Comparison of results from glucose tolerance test (GTT) in the different groups revealed a significant difference 15 and 60 min (AUC) following the glucose bolus, with stressed animals displaying higher levels of blood glucose compared to all the other groups ($p < 0.05$) (**Figure 3.4A**); glucose tolerance remained stable when pioglitazone was administered to CON and STR mice. Additionally, there was a significant treatment x time interaction ($F_{12,143} = 2.07$, $p = 0.022$).

Consistent with the results obtained in the GTT, animals receiving chronic stress showed a delayed response to insulin (**Figure 3.4B**) as compared to controls ($p < 0.05$). Additionally, STR+PIO mice showed a stronger response to insulin ($p < 0.05$), when compared to STR (30' and 60') (**Figure 3.4B**). A delayed response to insulin was also observed in STR+PIO- *vs.* PIO-treated animals ($p < 0.05$) (**Figure 3.4B**).

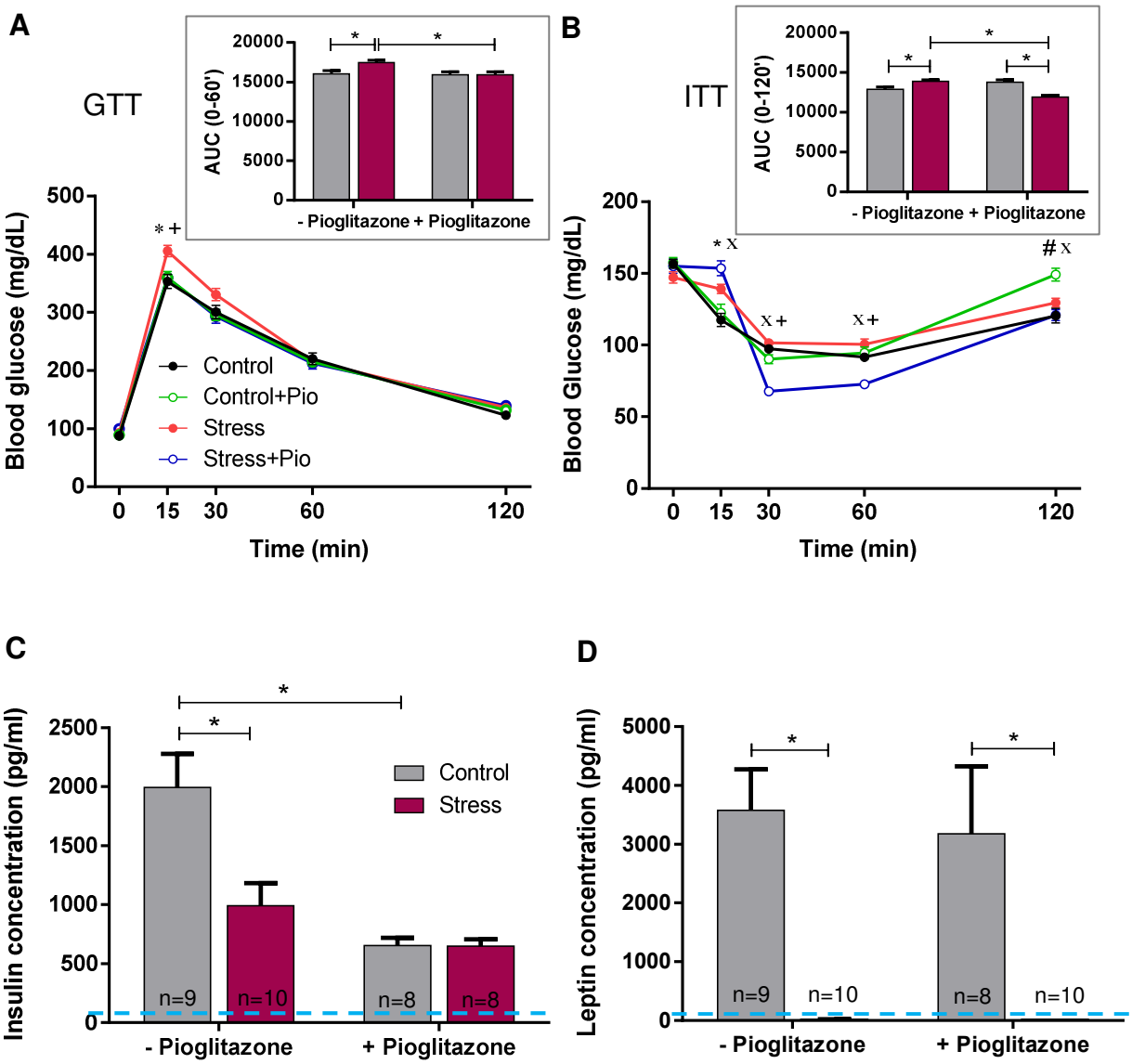


Figure 3.4. Metabolic status of mice exposed to chronic unpredictable stress (CUS) and the modulatory influence of pioglitazone. Blood glucose levels in CON (n=16), STR (n=16), PIO (n=15) and STR+PIO (n=15) groups were determined in mice challenged with a i.p. bolus of **(A)** glucose (2 g/kg) in the glucose tolerance test (GTT) or **(B)** insulin (0.75 U/kg) in the insulin tolerance test (ITT). Tests were performed in animals that had been previously fasted overnight (GTT) or for 6 h (ITT). Insets show areas under the curve (AUC) for 0-60 min (GTT) and 0-120 min (ITT). *, +, x, # indicate statistical difference ($p < 0.05$) between: * CON *vs.* STR; # CON *vs.* PIO; + STR *vs.* STR+PIO; x PIO *vs.* STR+PIO. Serum insulin **(C)** and leptin **(D)** levels at the end of the experiment in CON, STR, PIO and STR+PIO; number of animals is indicated on graphs. Blue dotted lines indicate minimum detectable concentrations of insulin (14 pg/ml) and leptin (19 pg/ml), respectively. Data are shown as mean ± SEM, * indicates $p < 0.05$.

Basal levels of plasma glucose were restored earlier (120') in the PIO-treated than in the CON and STR+PIO-treated mice ($p < 0.05$) (**Figure 3.4B**). There was a significant treatment x time interaction ($F_{12,145} = 11.12$, $p < 0.0001$). Serum levels of insulin at the end of the study revealed significant (suppressive) effects of STR and PIO ($p < 0.05$) (**Figure 3.4C**). Serum levels of leptin were significantly reduced to below detection limits of the assay in STR- and STR+PIO- treated mice ($p < 0.05$; **Figure 3.4D**).

In summary, stressed animals showed impaired glucose tolerance and insulin sensitivity with parallel reductions in serum insulin and leptin levels.

Stress-induced hyperlocomotion is not reversible by PIO treatment

Locomotor activity plays an important role in determining a variety of motivational and cognitive behaviours, and its measurement also provides an indirect index of general health. Results of the open field test (OF) used to monitor locomotor activity are shown in **Figure 3.5**. STR group displayed hyperlocomotor behaviour as compared to controls ($p < 0.05$), an effect that was not reversed by PIO treatment. In particular, the total distance travelled (**Figure 3.5A**) and the number of line crossings (**Figure 3.5B**) were significantly higher ($p < 0.05$) in STR and STR+PIO groups *vs.* controls.

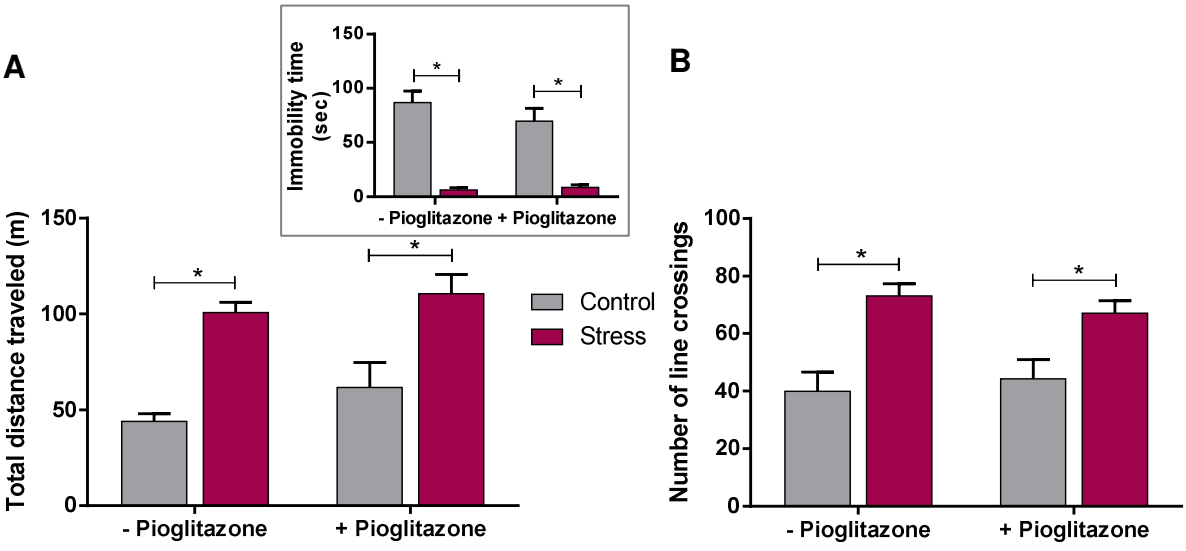


Figure 3.5 Pioglitazone does not reverse stress-induced hyperactivity in mice. An open field test was used to evaluate **(A)** Total distance travelled and immobility time (*inset*) and **(B)** Number of line crossings in following groups: CON (n=16), STR (n=16), PIO (n=15) and STR+PIO (n=15). Data are presented as mean ± SEM, * indicates $p < 0.05$.

The hyperactivity of STR mice was also evident from their significantly low immobility time ($p < 0.05$) when compared to controls (**Figure 3.5A**; *inset*); this measure in STR mice was not counteracted by PIO treatment.

Influence of stress and pioglitazone on motivation to retrieve a food reward

Motivation plays an essential role in cognitive processes such as learning and memory, including conditioned responses to appetitive stimuli (food rewards); the latter is particularly relevant in the context of the observed effects of stress and PIO on metabolism, as reported in the preceding sections. Further, learning and memory are cognitive processes of high relevance to Alzheimer's disease, a central focus of this thesis. Motivation was tested over 3 days (1 session/day, 30 min each), during which animals received 15 presentations of a liquid food reward which they could retrieve without "working" for it.

Activity, measured as beam breaks per minute was comparable between all groups (**Figure 3.6A**) overall, the rate of beam-breaking decreased over successive sessions (Time effect: $F_{2,48} = 35.90$, $p < 0001$). Overall, all groups of mice (CON, STR, CON+PIO and STR+PIO) learnt the rules of the motivation test in so far that they showed progressive reductions in the latency (Ovetall treatment effect: $F_{3,49} = 6.67$, $p = 0.001$; time effect: $F_{2,48} = 56.23$, $p < 0.0001$) and time to finish the session (Treatment x time interaction: $F_{6,96} = 2.13$, $p = 0.056$) (**Figure 3.6B** and **C**); they also showed a tendency to make a higher number of entries into the food tray during each subsequent test session (Treatment x time interaction: $F_{6,96} = 2.78$, $p = 0.015$) (**Figure 3.6D**). Closer analysis of the results showed that STR and CON mice did not differ on any of these parameters (**Figure 3.6B-D**). However, as compared to CON, the CON+PIO group were less motivated to retrieve the appetitive reward during at least one test session [greater latency ($p < 0.05$) and time to complete the task ($p < 0.05$) during sessions 2 and 3, **Figure 3.6B** and **C**, respectively; reduced number of food tray entries during session 2, **Figure 3.6D**]. Interestingly, motivation for the food reward was increased in the STR+PIO (*vs.* PIO) during the third (last) test session in terms of both, latency and total time taken to finish the session (**Figure 3.6B** and **C**; $p < 0.05$).

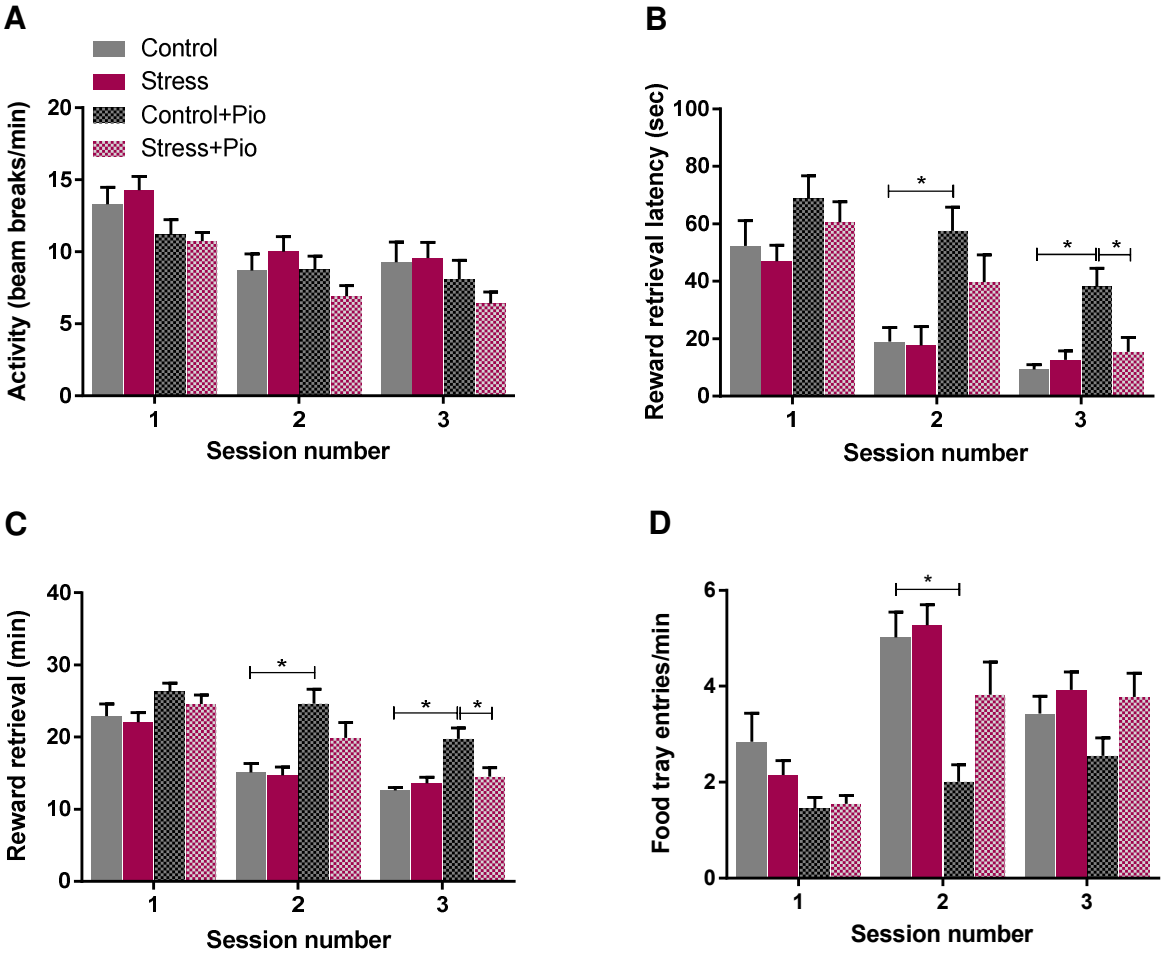


Figure 3.6. Motivation for a food reward is decreased by pioglitazone treatment but not by stress. Mice received 15 liquid food reward (condensed milk with 14% sugar) presentations at a variable interval of 10-40 sec. **(A)** Activity (beam breaks/min), **(B)** Reward retrieval latency (sec), **(C)** Time to retrieve reward (min) and **(D)** Food tray entries/min were analyzed in CON (n=15), STR (n=16), PIO (n=11) and STR+PIO (n=13) groups of mice, that have been food restricted. The test was carried out over 3 days. Data are shown as mean ± SEM, * indicates $p < 0.05$. Only data from animals reaching predefined criteria (see Materials & Methods) are depicted and included in the statistical analyses.

Operant conditioning, a task that measures both motivation and associative learning, requires "working to receive a reward". Here, mice had to nose-poke to receive an appetitive (food) reward, after a light stimulus appeared on the touchscreen. The test consisted of 20 trials (each lasting for a maximum of 45 min) per day, the criterion being that mice completed 20 trials within 20 min on at least 3 consecutive days.

All treatment groups (CON, STR, CON+PIO and STR+PIO) showed a progressive increase in activity (beam breaks) when presented with the task during 8 independent test sessions (**Figure 3.7A**); a significant treatment-group x time effect was found ($F_{21,315}$ = 1.80, p = 0.01). Moreover, all groups showed gradual improvements in acquisition of the appetitive learning task, measured as number of completed trials per session (**Figure**

3.7B); Treatment x time interaction: $F_{21,315} = 3.12$, $p < 0.0001$), time to complete each session (**Figure 3.7C**); $p < 0.0001$; overall treatment effect: $F_{3,44} = 6.12$, $p = 0.001$; time effect: $F_{7,308} = 118.49$, $p < 0.0001$) and number of stimulus touches (nose-pokes) (**Figure 3.7D**). Specifically, the CON and STR groups showed identical rates of learning the operant task, with approximately 94% (15/16) of the mice in each group reaching criterion (**Figure 3.7**). In contrast, as judged on the basis of all parameters (**Figure 3.7B-D**), CON+PIO animals displayed poor operant learning (53% or 8/15 mice reached criterion; $p < 0.05$ *vs.* respective results in CON mice) while mice concomitantly exposed to STR and PIO (STR+PIO) proved to be slightly better learners (73% or 11/15 mice reached criterion; $p < 0.05$ *vs.* respective results in CON+PIO mice) (**Figure 3.7B-D**).

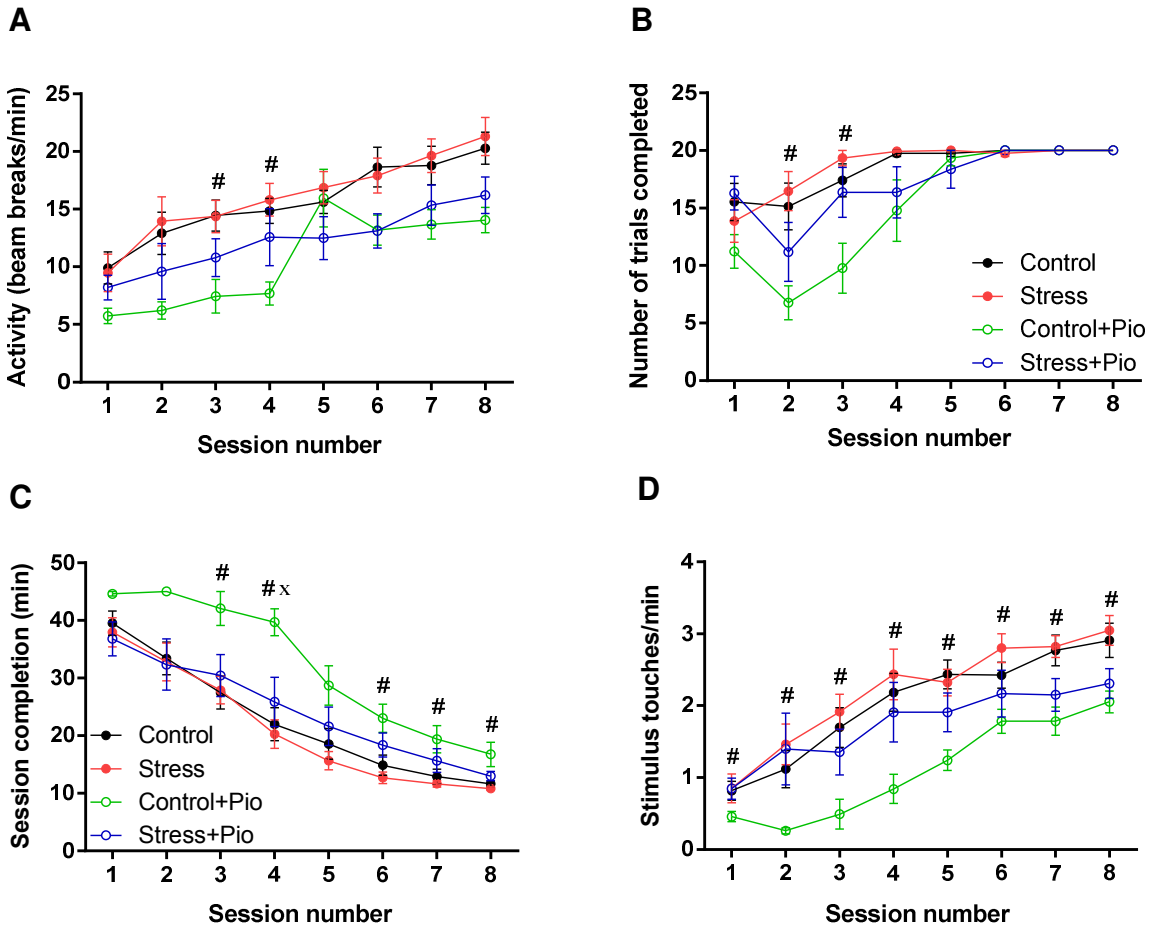


Figure 3.7. Pioglitazone but not stress impairs associative learning for an appetitive reward. Mice received 20 light stimuli at the center of the touchscreen at a variable interval of 10-40 sec. Liquid food reward (condensed milk with 14% sugar) was delivered with each "nose-poke" of the touchscreen. The test was carried out over 8 daily sessions. **(A)** Activity (beam breaks/min), **(B)** Number of trials completed, **(C)** Time to finish session (min) and **(D)** Stimulus touches/min. For the test, mice were food restricted; CON (n=15), STR (n=15), PIO (n=9) and STR+PIO (n=11) groups of mice. Data are shown as mean ± SEM, # indicates statistical difference ($p < 0.05$) between CON and PIO groups; x indicate statistical difference ($p < 0.05$) between PIO and STR+PIO groups. Only data from animals reaching predefined criteria (see Materials & Methods) are depicted and included in the statistical analyses.

Taken together, our results indicate that pioglitazone impairs operant learning in non-STR mice > in STR mice. Nevertheless, interpretation of these findings may be confounded by the fact that CON+PIO treatment is characterized by reduced motivation (**Figure 3.6B-D**) and increased body weight and adiposity (**Figure 3.3A-D**), i.e. the result may not necessarily demonstrate that PIO induces learning deficits in non-STR mice but rather reflect the lowered motivation of overweight animals to learn to work for an appetitive reward (food).

Potential of pioglitazone to prevent cognitive decline induced by CUS

The novel object paradigm which is memory-dependent on the cortex and hippocampus (Barker and Warburton, 2011; Warburton and Brown, 2015) was used to examine whether PIO can counter the negative impact of STR on memory. The test comprises two parts, one which monitors object location (OLR), a hippocampus-dependent task and the other which measures novel object recognition (NOR), a task that depends on the perirhinal cortex (Barker and Warburton, 2011; Warburton and Brown, 2015).

Stress appeared to decrease recognition memory in the object location recognition task (**Figure 3.8A**). However, the stress-effect does not reach significance due to high variability among the group. Object recognition memory was comparable between all the groups (**Figure 3.8B**).

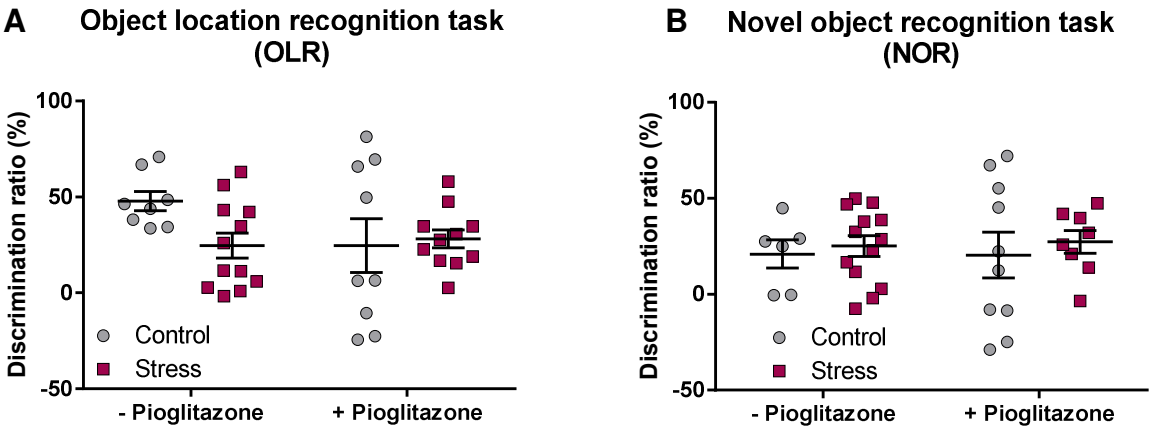


Figure 3.8. Object location and recognition paradigm in mice after CUS and pioglitazone treatment. **(A)** Object location recogntion task in CON (n=10), STR (n=12), PIO (n=9) and STR+PIO (n=11) mice: 1 h after the familiarization to the objects, one object was moved to a novel location. **(B)** Novel object recognition test in CON (n=6), STR (n=12), PIO (n=9) and STR+PIO (n=8) mice: 24 h after the familiarization phase one of the objects was replaced with a novel object. Animals were allowed to explore the objects for 10 minutes in both tests. Respective discrimination ratios were computed as described in Materials and Methods. Data are presented as mean ± SEM, * indicates $p < 0.05$. Only data from animals reaching predefined criteria (see Materials & Methods) are depicted and included in the statistical analyses.

Interestingly, PIO mice tent to show a two directional behaviour; although, pioglitazone decreased novel location- (**Figure 3.8A**) and recognition (**Figure 3.8B**) memory in a subgroup of animals, another subgroup of PIO mice showed an increase in their recognition memory.

Aberrant phosphorylation of tau protein: interactions between stress and pioglitazone

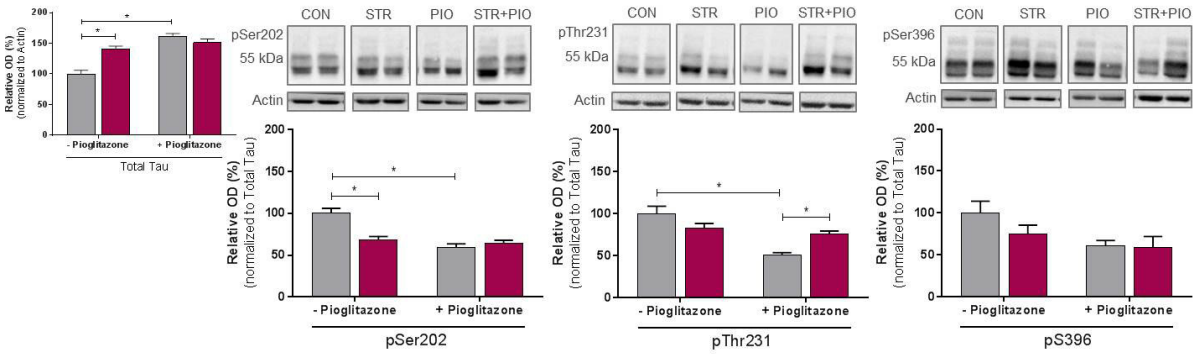
In light of previous work from our laboratory that showed that CUS can induce hyperphosphorylation of tau at epitopes associated with AD pathology (Catania *et al*., 2009; Sotiropoulos *et al*., 2011) and the results reported in the previous section, we next examined whether stress and/or pioglitazone influence the phosphorylation of tau. Our analysis focused on the prefrontal cortex (PFC) and dorsal and ventral hippocampus; the PFC and dorsal hippocampus are strongly implicated in cognitive functions (Wall and Messier, 2001; Samson and Barnes, 2013; Strange *et al*., 2014) and are "victims" of amyloid and tau pathology in AD brains (Catania *et al*., 2009; Sotiropoulos *et al*., 2008b; Mu and Gage, 2011; Sotiropoulos *et al*., 2011; Braak and Del Tredici, 2015; Lopes *et al*., 2016) in contrast, the ventral hippocampus is generally associated with emotional processing and neuroendocrine regulation (Fanselow and Dong, 2010). The reason for focussing only on tau was that rodents do not normally develop amyloid pathology. Here, tau and its aberrantly phosphorylated epitopes (phosphorylation at Thr 231, Ser 202 and Ser 396), was monitored using immunoblotting of prefrontal cortical and dorsal and ventral hippocampal lysates prepared from the CON-, STR-, CON+PIO- and STR+PIO-treated mice, following their behavioural and endocrine testing at the termination of the whole experiment.

As compared to CON, STR-treated mice showed increased expression of total tau in the prefrontal cortex and ventral hippocampus (*insets*, **Figure 3.9A** and **C**, $p < 0.05$). Total tau levels were also increased in the prefrontal cortex of PIO-treated mice (relative to CON mice; *inset,* **Figure 3.9A**; $p < 0.05$) and in the ventral hippocampus of STR+PIO mice (relative to CON+PIO mice; *inset,* **Figure 3.9C**; $p < 0.05$). None of the treatments influenced the expression of total tau in the dorsal hippocampus (*inset,* **Figure 3.9B**).

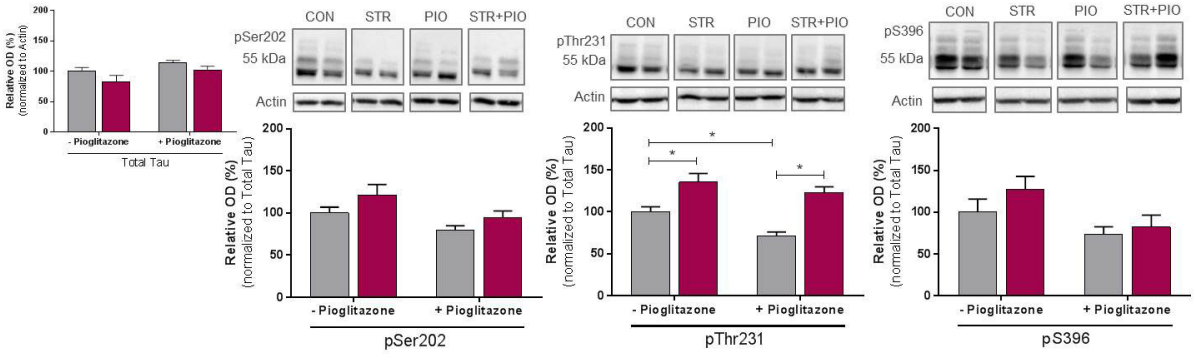
The various treatments exerted differential, epitope-specific effects on the phosphorylation of tau in the prefrontal cortex, dorsal and ventral hippocampus, as shown

in **Figure 3.9A-C**, respectively. Tau was found to be less phosphorylated at Ser202 only in the prefrontal cortex of STR mice (**Figure 3.9A**; $p < 0.05$); as compared to CON, PIO treatment (CON+PIO group) resulted in a significant reduction in levels of tau that was phosphorylated at the Ser202 epitope (**Figure 3.9A**; $p < 0.05$).

A. Prefrontal cortex



B. Dorsal hippocampus



C. Ventral hippocampus

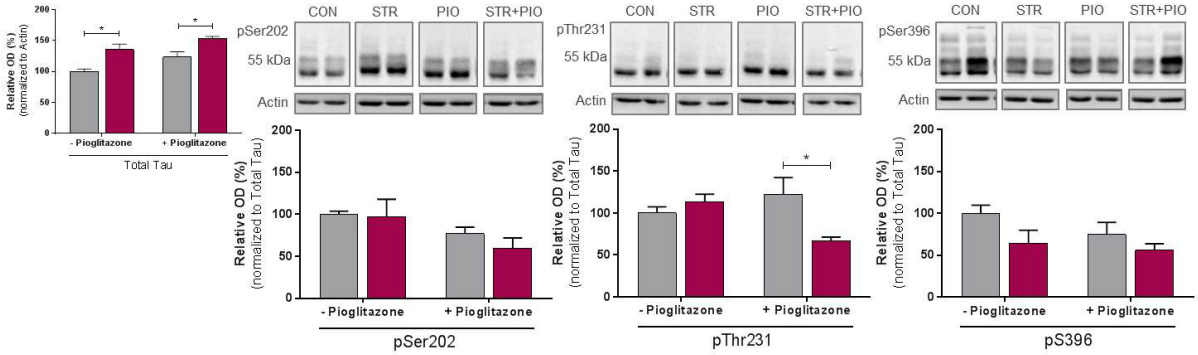


Figure 3.9. Effect of CUS+CMS and pioglitazone on tau phosphorylation at Serine 202 (pSer202), Threonin (pThr231) and Serine 396 (pS396) in (A) Prefrontal cortex, **(B)** Dorsal hippocampus, and **(C)** Ventral hippocampus, of CON (n=7), STR (n=6), PIO (n=7) and STR+PIO (n=7) groups. Phosphorylated tau has been normalized to total-tau. Data are shown as mean ± SEM, * indicates $p < 0.05$.

Levels of tau-pThr231 were decreased in the prefrontal cortex of mice treated with PIO (CON+PIO group) *vs.* CON mice (**Figure 3.9A**; $p < 0.05$). An increase in the levels of tau-pThr231 was observed in the dorsal hippocampus of STR *vs.* CON mice (**Figure 3.9B**; $p < 0.05$), whereas levels of this phosphorylated epitope were decreased in CON+PIO *vs.* CON mice (**Figure 3.9B**; $p < 0.05$). The prefrontal cortex and dorsal hippocampus of STR+PIO mice showed significantly higher levels of tau-pThr231 when compared to the corresponding areas in CON+PIO mice (**Figure 3.9A** and **B, respectively**; $p < 0.05$ in both cases). However, ventral hippocampal expression of tau-pThr231 was reduced in STR+PIO *vs.* CON+PIO mice (**Figure 3.9C**; $p < 0.05$).

None of the treatments had any significant influence over the expression of tau-pSer396 in any of the brain areas (prefrontal cortex, dorsal and ventral hippocampus) studied (**Figure 3.9A-C**).

Taken together, our results show that STR increases the expression of total tau in the prefrontal cortex and ventral hippocampus. In general, STR tends to regulate tau phosphorylation (at either Ser202 or Thr231) in opposite directions in the prefrontal cortex (decreased) and dorsal hippocampus (increased). While PIO reduces levels of tau-Thr231 phosphorylation in CON animals in the prefrontal cortex and dorsal hippocampus, it does not alter the expression of tau or of its hyperphophorylated forms in stressed mice.

3.4 Discussion

The aim of this study was to examine how the PPARγ agonist PIO influences the metabolic and cognitive outcomes of chronic stress. PPARγ agonists have been shown to reduce the response to stress (Ulrich-Lai and Ryan, 2013) and have also been suggested to protect against a variety of stress-related disorders of the brain, including stroke, depression and AD (Heneka and Landreth, 2007; García-Bueno *et al*., 2010; Zolezzi *et al*., 2014; Pérez and Quintanilla, 2015). Here, we used an established chronic unpredictable stress (CUS) paradigm which is known to induce cognitive impairments (Sousa *et al*., 2000; Cerqueira *et al*., 2007a) and AD-like pathology in association with mnemonic deficits (Catania *et al*., 2009; Sotiropoulos *et al*., 2011; Lopes *et al*., 2016). Potential confounds were considered when planning the present experiments, which included a long series of physiological and behavioural analyses. On the one hand, we

considered the possibility that CUS on the day of behavioural testing would interfere with the timely and appropriate execution of daily stress procedures, but also with the outcome of behavioural testing. On the other, we considered previous reports that demonstrated that the effects of CUS wane with time after CUS has been terminated (Sousa *et al*., 2000). In an attempt to overcome these constraints, mice were initially exposed to CUS for 6 weeks before being exposed to a milder stress (chronic mild stress, CMS) for a further 6 weeks during which time the majority of physiological and behavioural evaluations were conducted.

The CUS protocol used proved efficient insofar that it increased basal levels of corticosterone (CORT) secretion and blunted the night-day ratio in stressed (STR) mice. Further, CUS caused a loss in body weight (BW) and increased locomotor activity (as measured in the open field) in CON animals. Contrary to expectations, while PIO accentuated the CORT response to chronic stress (CUS, STR), STR reduced the night-day ratio of CORT levels in STR+PIO mice. The latter result is interesting because, while higher levels of cortisol *per se* are associated with poor cognitive functioning (Lee *et al*., 2007) higher diurnal ratios correlate positively with higher cognitive functioning (Geerlings *et al*., 2015; Johar *et al*., 2015). Thus, stimulation of PPARγ with PIO leads to a cortisol profile (CORT levels) that would be expected to predict poorer, rather than better, cognitive performance. As will be discussed in detail below, this view is supported by our own behavioural observations. In light of other studies that suggest that PPARγ agonists may have indirect pro-cognitive activity (Pedersen *et al*., 2006; Escribano *et al*., 2009; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Rodriguez-Rivera *et al*., 2011; Denner *et al*., 2012; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Jahrling *et al*., 2014; Chen *et al*., 2015; Yu *et al*., 2015), the present finding needs further replication, extensive dose-response testing, and evaluation of cognitive behaviour using a broad test battery.

It has been reported that exposure of rats to acute restraint stress, results in an upregulation of PPARγ expression in the cortex (García-Bueno *et al*., 2005a; García-Bueno *et al*., 2008a), an area also involved in the regulation of the neuroendocrine response to stress (Ulrich-Lai and Ryan, 2013; Herman *et al*., 1996). This would suggest that changes in PPARγ expression are subject to regulation by glucocorticoids (GC, e.g. CORT) released during stress and/or represent an adaptive response to stress. The latter possibility seems highly likely in view of the present finding that PIO treatment

attenuated the CORT response of CON and STR mice to an acute stressor that was delivered at the end of the experiment. Our finding is consistent with that of Ryan *et al*. (2012) who reported that rats pre-treated with another TZD (rosiglitazone, 5 d) before exposure to acute restraint stress displayed an attenuated CORT response to the stressor. On the other hand, it should be noted that PIO treatment failed to counter STR-induced hyperlocomotion.

PPARγ is a master regulator of adipogenesis, adipocyte differentiation and fatty acid storage (Lehrke and Lazar, 2005; Cho *et al*., 2008; Tontonoz and Spiegelman, 2008; Wahli and Michalik, 2012; Ahmadian *et al*., 2013). Activation of PPARγ by TZDs such as PIO increases insulin sensitivity and TZDs have been promoted as anti-diabetic agents (Hofmann and Colca, 1992; Nolan *et al*., 1994; Lehmann *et al*., 1995; Willson *et al*., 2000; Berger and Moller, 2002). Since overweight and obesity are considered risk factors for T2D, it is somewhat paradoxical that TZDs are known to cause BW gain (Lehrke and Lazar, 2005; Cariou *et al*., 2012; Ahmadian *et al*., 2013; Soccio *et al*., 2014; Sauer *et al*., 2015). This property is reflected in the current study where PIO was found to reverse STR-induced BW loss but led to increased BW in CON (non-STR) animals. Interestingly, WAT mass did not differ between CON and STR mice and whereas PIO treatment resulted in higher WAT mass in CON mice, PIO did not alter WAT mass in STR animals. These findings indicate the likely complex interactions and mechanisms that underlie the effects of PIO on BW and WAT mass in CON and STR animals. Resolving these would be important because lower lean mass, more than high BW, appears to be causally related to cognitive dysfunction (Burns *et al*., 2010; Pasha *et al*., 2016). Adding to the difficulty of interpreting the obtained results stems from the fact that, while STR and GC are catabolic and cause a loss of lean mass, GC exert differential influences over BW and WAT mass that are difficult to unravel and which depend on the physiological context (Asensio *et al*., 2004; Wake and Walker, 2004; Seckl and Walker, 2004; Wang, 2005; Lee *et al*., 2014; Razzoli and Bartolomucci, 2016). In recent years WAT has been recognized as a highly active metabolic and endocrine metabolic tissue. For example, WAT expresses the enzyme 11β-hydroxysteroid-dehydrogenase type 1 (11β-HSD1), which converts inactive cortisone into active cortisol in humans (or corticosterone in rodents), not only increases GC availability but also contributes to metabolic disturbances including glucose intolerance and insulin resistance (Asensio *et al*., 2004; Wake and Walker, 2004; Seckl and Walker, 2004; Wang, 2005); in turn, the latter two conditions

will give rise to an accumulation of fat and obesity. Dissection of the relationship between GC/STR and metabolism and their regulation by PIO will therefore require a stepwise analysis of this complex vicious cycle.

Results presented here are largely consistent with previous reports that STR impairs glucose metabolism and insulin sensitivity (Rasgon and Kenna, 2005; Zardooz *et al*., 2006; Depke *et al*., 2008; Rostamkhani *et al*., 2012; Ghalami *et al*., 2013; Li *et al*., 2013; Detka *et al*., 2013; Hwang and Weiss, 2014). Moreover, as expected, PIO treatment improved glucose tolerance and insulin sensitivity; the latter was also evident from the lower plasma levels of insulin found in PIO-treated CON and STR mice. Inexplicably, blood insulin levels were reduced after acute stress in mice despite the impaired glucose metabolism/insulin sensitivity displayed by these mice. Levels of leptin (a peptide hormone released from adipocytes, which together with insulin, signals satiety - Maniam and Morris, 2012; Sominsky and Spencer, 2014) were also measured at the end of the experiment, immediately following exposure to an acute stressor. Leptin levels were decreased in stressed mice irrespective of whether they had received PIO or not. Interestingly, leptin secretion was previously reported to be elevated after acute stress (Konishi et al., 2006; Maniam and Morris, 2012; Tomiyama *et al*., 2012) and also following corticosterone treatment (Karatsoreos *et al*., 2010). However, other studies using chronic stress paradigms observed bidirectional effects on leptin, with chronic social defeat stress reducing plasma leptin levels, possibly due to a depletion of leptin in white adipose tissue (Iio *et al*., 2014), while chronic heat stress increased leptin in plasma and subcutaneous WAT (Morera *et al*., 2012). At least some of these conflicting results may be explained by alterations in the fine balance between GC and leptin levels and the sensitivity of the brain (primarily hypothalamus) to leptin (Sominsky and Spencer, 2014). The present finding of low leptin and insulin levels in stressed animals suggests central resistance to the actions of these peptides, resulting in hyperphagia and eventually (after CUS), weight gain.

Overweight and obesity, including high adiposity, results from eating in excess of actual energy requirements. In an attempt to explore whether STR and PIO influence behavioural motivation to obtain an appetitive (food) reward, we conducted two sets of experiments. In the first, parameters providing information on motivation of food-deprived mice to retrieve a palatable liquid diet was assessed. The second experiment involved an operant learning paradigm in which the animals needed to learn/memorize

the task which involved working towards receiving a food reward. Results of both tests showed that whereas motivation to eat was not altered in the STR group, PIO administration to CON (non-STR) mice lowered motivation to eat. It is important to note that, despite their differing body masses, none of the treatment groups differed in their locomotor activity, an important factor in tests of this nature. However, interpretation of the findings is potentially confounded by the facts that PPARγ is closely involved in the regulation of energy metabolism/availability and that motivation was here judged in terms of retrieval of a food (energy-delivering) reward. Interestingly, we found that mice that received STR+PIO showed slightly increased motivation/learning, a finding that may reflect the greater energy needs created by STR (a catabolic stimulus) but also the reportedly better behavioural performance that correlates with elevated CORT levels following touchscreen training (Mallien *et al*., 2016); while it could be argued that all treatment groups shared the latter experiences, one cannot exclude interactions introduced by the CUS/CMS and PIO-treatment paradigms which themselves exerted effects on metabolism and possibly behaviour. Given that PIO caused an elevation in BW, it is relevant to note here that our group previously showed that body weight affects motivation and appetitive learning (Harb and Almeida, 2014) insofar that overweight and obese mice display reduced motivation (and appetitive learning) in such tests (Harb and Almeida, 2014). In order to cast more light on this issue, an additional experiment, described in Chapter 4, was conducted.

As mentioned before, chronic STR produces cognitive deficits and is well exemplified by impairments in hippocampus-dependent spatial memory and prefrontal cortex-dependent behavioural flexibility (Cerqueira *et al*., 2007a; Catania *et al*., 2009; Sotiropoulos *et al*., 2011; Lopes *et al*., 2016). Such behaviours, based on the Morris water maze test (Shenk and Morris, 1985) have been extensively evaluated in animal models of AD and to test the efficacy of supposed pro-cognitive or AD-modifying drugs (Anger, 1991). More recently, these tests have been complemented by the novel object recognition (NOR) test (Baker and Kim, 2002; Eagle *et al*., 2013; Baglietto-Vargas *et al*., 2015) and, to some extent, the object location recognition (OLR) test (Lopes *et al*., 2016). The advantage of these newer tests over the tests of spatial memory using the Morris water maze, are that: i) they do not involve swimming (water is considered aversive to mice); ii) while both the OLR and NOR tests have strong spatial components, they also require visual and

olfactory skills, for example – a point that is important when one considers that memory deficits in AD occur in domains that extend beyond spatial memory.

While less is known about the detrimental effects of STR on OLR (e.g. Lopes *et al*., 2016), NOR has been consistently been reported to be impaired by STR in both, rats (Baker and Kim, 2002; Ivy *et al*., 2010; Eagle *et al*., 2013) and mice (Tsukahara *et al* 2015). Our studies showed that STR reduces OLR memory, albeit non-significantly due to high inter-individual variability. We also observed a tendential reduction of this impairment in STR mice that received PIO treatment. The aforementioned high inter-individual variability may have also contributed to our failure to observe significant STR-induced deficits in OLR (cf. Lopes *et al*., 2016) and, likewise, to the lack of changes in OLR and NOR performance in PIO-treated mice. The failure to see pro-mnemonic effects of PIO goes contrary to previous reports (Pedersen *et al*., 2006; Escribano *et al*., 2009; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Rodriguez-Rivera *et al*., 2011; Denner *et al*., 2012; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Jahrling *et al*., 2014; Chen *et al*., 2015; Yu *et al*., 2015). In light of the fact that PIO causes BW increases in association with adiposity (seen in this and previous studies), it deserves mentioning that obesity is known to impair OLR in mice (Heyward *et al*., 2012; Valladolid-Acebes *et al*., 2013) in parallel changes in hippocampal expression of genes linked to memory consolidation (Heyward *et al*., 2012). In addition, the negative impact of PIO on motivation described above (albeit with respect to motivation for food), should also be considered as a contributory factor to the observed inability of PIO to promote cognitive behaviour.

Despite the unclear behavioural results obtained, our interest to examine whether (and how) TZDs such as PIO may exert their positive actions on cognition and AD ((Pedersen *et al*., 2006; Escribano *et al*., 2009; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Rodriguez-Rivera *et al*., 2011; Denner *et al*., 2012; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Jahrling *et al*., 2014; Chen *et al*., 2015; Yu *et al*., 2015) led us to search for possible cellular correlates of PIO action. To this end, we considered assaying hippocampal and frontocortical proteins that are not only implicated in the regulation of cognition, but also AD. Two proteins that fulfill this criteria are amyloid β (Ittner and Götz, 2011; Selkoe & Hardy, 2016) and the cytoskeletal protein tau (Iqbal *et al*., 2010; Spillantini and Goedert, 2013; Wang and Mandelkow, 2016). Examination of Aβ was

neglected because murine Aβ is not assayable using immune-based technologies. Tau, which serves to stabilize microtubules, is highly abundant in the central nervous system (CNS), being found in neuronal axons and dendrities, astroglia and microglia cells (Gu *et al*., 1996; Lee *et al*., 2001); in the last few years, tau has been described to play an important role in synaptic plasticity (Hoover *et al*., 2010; Ittner *et al*., 2010; Kimura *et a*l., 2010; Sotiropoulos *et al*., 2011; Kimura *et al*., 2013). The cytoskeletal and synaptic functions of tau are compromised and lead to the so-called tauopathies, of which AD is one, when the protein becomes aberrantly hyperphosphorylated at certain serine and threonine sites, through the activity of kinases such as glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (cdk5) (Lee *et al*., 2001; Takashima, 2006; Lei *et al*., 2011; Shukla *et al*., 2012; Papadopoulou *et al*., 2015). Phosphorylation of sites such as Ser202, Thr231 and Ser396 leads to the oligomerization and aggregation of tau, and the formation of neurofibrillary tangles (Lee *et al*., 2001; Augustinack *et al*., 2002; Lauckner *et al*., 2003; Hampel *et al*., 2005; Spillantini and Goedert, 2013; Wang and Mandelkow, 2016), which, besides Aβ plaques, represents a neuropathological hallmark of AD (LaFerla and Oddo, 2005; Iqbal *et al*., 2010; Holtzman *et al*., 2011; Ittner and Götz, 2011; Selkoe and Hardy, 2016; Sotiropoulos and Sousa, 2016; Wang and Mandelkow, 2016). Our lab previously reported that chronic stress leads to hyperphosphorylation of tau in the hippocampus and prefrontal cortex, accompanied by deficits in cognitive behaviour (specifically, spatial memory and reversal learning) (Sotiropoulos *et al*., 2011). More recently, studies in mice demonstrated that stress cannot induce tau pathology or cognitive impairment if the tau gene is deleted (Lopes *et al*., 2016).

Results from the present study show that STR upregulates the expression of total tau in the prefrontal cortex and ventral hippocampus, thus increasing substrate availability for kinase-mediated hyperphosphorylation. Further, we found that treatment effects (STR, PIO or STR+PIO) occurred in epitope- and region-specific manners. Although our results did not faithfully reproduce all the changes reported in the above-mentioned earlier studies from our laboratory, STR-induced increases in tau-pThr231 of the dorsal hippocampus would appear to be a robust response; interestingly, in the present experiments, stress was seen to reduce the levels of tau-pThr231 in the ventral hippocampus of STR+PIO mice. Importantly, PIO decreased the levels of pThr231 in the cortex and dorsal hippocampus of control, but not STR, mice. Several considerations may

help explain these disparate findings. First, interpretation of immunoblotting of lysates is based on the assumption that the assays are sufficiently sensitive to detect very small (but functionally significant) changes in a relatively large tissue extract. Second, capturing phosphorylation is notoriously difficult because, at least initial phosphorylation events tend to be dynamic "on-off" events. Lastly, and because of the previous fact, obtaining reproducible results strongly depends on tissue preparation and their rapid and appropriate storage, as well as tissue thawing and lysis for subsequent analysis (even if phosphatase inhibitors are employed as was the case here). The large number of animals and analytes generated by this study may well have presented a confound despite care at all stages of the collection and analytical procedures. In this study, multiple samples had to be assayed in a single run because of the inherent limitations of such assays.

In summary, the main findings of this series of experiments are that PIO can modify the endocrine and metabolic dysfunction that results from chronic STR exposure. Strikingly, while PIO counteracted many of the metabolic maladaptations associated with STR, the changes were not necessarily accompanied by predictable alterations in the endocrine response to STR. Further, PIO elicited changes in motivation and memory but also in the phosphorylation status of tau. Altogether, the results obtained here indicate complex STR x Endocrine x Metabolic x PIO interactions, the dissection of which will require further series of studies. Those studies will also require detailed temporal analyses of likely sequential events. Despite the inconclusive nature of the data obtained, the various tendential changes observed do not outrightly dismiss the underlying hypothesis that PPARγ activation may be a useful strategy to maintain metabolic and cognitive health, or that drugs such as PIO may help prevent the decline of cognitive health due to the battery of stressful and metabolic insults experienced over lifetime. In future, it will be important to establish dose-response curves for each of the parameters investigated. Choice of dose and route of administration in the present work was based on previous reports (Heneka *et al*., 2005; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Chen *et al*., 2015) but the possibility that dosage was sub-optimal cannot be excluded, especially when searching for central effects since it is estimated that only 18% of PIO penetrates the blood brain barrier (BBB) (Heneka *et al*., 2005).

CHAPTER 4

Does pioglitazone modulate motivation and hedonic preference?

Abstract

The peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear receptor that plays a critical role in the regulation of adipogenesis, lipid and glucose metabolism. PPARγ agonists that are effective anti-diabetic drugs have been recently shown to reduce motivation for opioid and alcohol consumption and to modulate dopamine transmission in the reward pathway, specifically in the nucleus accumbens and ventral tegmental area. Results presented in Chapter 3 indicated that pioglitazone treatment reduces motivation for a food reward. To examine whether pioglitazone affects a general motivational state or motivation for a specific stimulus (e.g. energy), mice were placed on pioglitazone treatment for 6 weeks and tested for motivation (using tests of "pure motivation" and instrumental learning). Our results show that the effect of pioglitazone on pure motivation and operant learning is strongly influenced by body weight (BW) (energy reserves): animals of higher BW were less motivated to retrieve a food reward than those with lower BW. The fact that pioglitazone itself leads to weight gain made it difficult to dissect whether the drug directly affects motivation or whether the effects observed are secondary to drug-induced increases in energy reserves; similarly, conclusions about the interaction between motivation and cognitive processes (e.g. learning) could not be easily reached. However, it was expected that an experimental design devised to distinguish between the role of hedonia (a contributant to motivational behaviour) from that of energy would begin to help resolve the inherent problems associated with such studies. For this, we monitored the preferential consumption of solutions containing sucrose (sweet, energy-rich) *vs.* saccharin (sweet, energy-free) *vs.* water alone in control and pioglitazone-treated (high BW) mice that were in a sated or fasted state. Unexpectedly, all mice showed strong preference for saccharin over sucrose (water was consumed minimally), irrespective of their energetic state. Interestingly, however, pioglitazone treatment decreased fluid consumption in general, but also the relative amount of saccharin intake in fasted animals. Together, these data suggest that PPARγ modulates motivation and its components, reward and hedonia, albeit through complex mechanisms that remain elusive at present.

4.1 Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear receptor, that heterodimerizes with the retinoid X receptor (RXR) to regulate the transcription of genes involved in adipocyte differentiation, fatty acid storage and glucose metabolism (Mangelsdorf *et al*., 1995; Berger and Moller, 2002; Tontonoz and Spiegelman, 2008; Harmon *et al*., 2011; Sauer, 2015; Lehrke and Lazar, 2005; Cho *et al*., 2008; Wahli and Michalik, 2012). Fatty acids are the endogenous PPARγ agonists (Xu *et al*., 1999, Berger and Moller, 2002; Tontonoz and Spiegelman, 2008; Harmon *et al*., 2011; Sauer, 2015), while the thiazolidinedione (TZD) class of drugs, which act as potent pharmacological ligands are implicated in the treatment of type 2 diabetes (T2D) because of their insulin-sensitizing properties (Hofmann and Colca, 1992; Nolan *et al*., 1994; Lehmann *et al*., 1995; Willson *et al*., 1996; Willson *et al*., 2000; Berger and Moller, 2002; Sauer, 2015).

Recently, the TZD pioglitazone (PIO) has been suggested to influence motivation and reward pathways, possibly finding an application in the treatment of drug addiction. Specifically, PIO was shown to diminish the rewarding properties of, and motivation for, heroin (de Guglielmo *et al*., 2015); the latter authors showed that PIO-induced reductions in dopamine levels in the nucleus accumbens (Acb), a key structure in the reward system [and decreased activity of dopaminergic neurons in the ventral tegmental area (VTA), which sends projections to the Acb] may explain this phenomenon (de Guglielmo *et al*., 2015). These results add support to previous work showing that PIO may also be useful in curbing excessive alcohol consumption (Stopponi *et al*., 2011, 2013); Importantly, the effects of PIO were abolished by central injections of the PPARγ antagonist GW9662 (Stopponi *et al*., 2011). Notably, PPARγ have been located in the Acb (Moreno *et al*., 2004) and VTA (Sarruf *et al*., 2009; de Guglielmo *et al*., 2015), key areas in the regulation of motivation, reward and pleasure (hedonia initated by cognitive, emotional, sexual and social stimuli) (Fields *et al*., 2007; Berridge and Kringelbach, 2013; Richard *et al*., 2013; Berridge and Kringelbach, 2015; Castro *et al*., 2015), including palatable food (Lowe and Butryn, 2007). As noted by Berridge and Kringelbach (2011, 2015), from an evolutionary perspective, anticipated pleasure motivates organisms to retrieve reward that promote survival; however, in today's human societies, such pleasures may lead to addictive behaviours. These authors (Berridge and Kringelbach 2011, 2013, 2015) argue

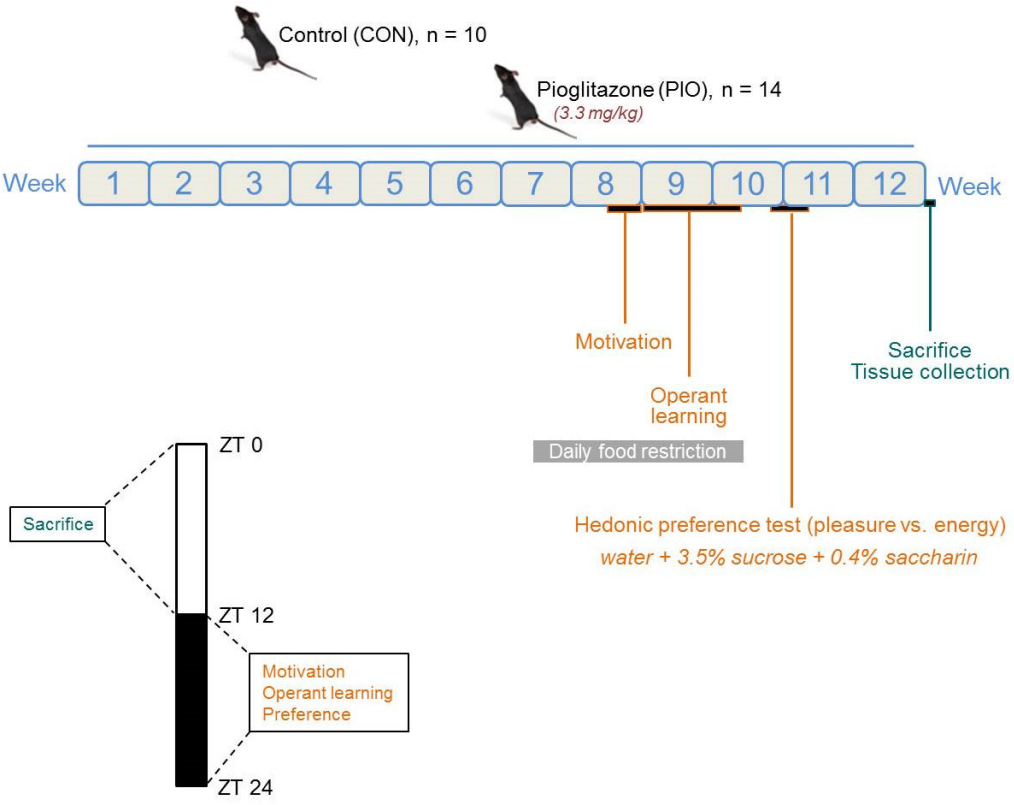
that the neuropsychological basis of reward perception involves 1) liking, derived from pleasure, 2) wanting, derived from the motivation to retrieve the reward, and 3) learning, a reward-dependent behaviour.

Results presented in Chapter 3 confirmed that PIO may have a negative influence over motivation to eat in mice, resembling its effects on reducing motivation for drugs and substances of abuse. We however considered that our observations may have been confounded by the fact that PIO causes an increase in body weight (BW) and subsequently higher energetic reserves. This reasoning was reinforced by our previous findings that overweight mice show lower motivation and appetitive learning skills than mice with normal BW (Harb and Almeida, 2014). Here, we performed three tests in an attempt to gain insight into the relationship between PIO-induced weight gain and eating behaviour. The first two tests compared how food-deprived mice retrieved a palatable reward *without* having to work (nose-poke) for it (pure motivation test) and after having to nose-poke (*work)* for the palatable reward (motivation tested in operant conditioning paradigm). The third test, also conducted in fed or fasted control and PIO-treated mice, sought to distinguish motivation to consume sucrose (a sweet, caloric drink) *vs.* saccharin (a sweet, calorie-free drink) *vs.* water; the concentration of the saccharin solution was adjusted so as to have the same hedonic (iso-hedonic) properties as the sucrose solution (cf. Young and Madsen, 1963; Beeler *et al*., 2012). Using these tests, we aimed to dissect out the hedonic and energetic components of food that may contribute to motivation for an appetitive stimulus and thereby to begin to understand the interaction between BW (as influenced by PIO) and motivation to eat. The importance of such a dissection was highlighted in a very recent paper which used a different test paradigm (Tellez *et al*., 2016).

4.2 Materials and Methods

Animals

Adult (4 months) male mice (C57/BL6 strain, Martinsried, Germany) were used in compliance with the European Union Council's Directive (2010/63/EU) and local regulations. Animals (n=24) were housed in groups (4 per cage) under standard laboratory conditions (temperature 22 °C; and relative humidity 50 ± 10%), in a reversed 12h light/12h dark cycle (lights on at 17:00). Unless specifically mentioned, animals had *ad libitum* access to food and water. A subgroup of animals (n=14) received pioglitazone (PIO; ActosTM Takeda Pharma A/S, Denmark) that was homogeneously mixed into their normal chow (see below) to provide each animal with a daily dosage of 3.3 mg PIO/kg BW (based on an average food consumption of 5 g of chow per animal/day). Control animals received normal chow (#1324 laboratory diet; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) throughout the experiment.



Scheme 4.1. Experimental design. A total of 24 mice were used in this experiment (10 controls, maintained on standard chow, 14 experimental animals which received PIO at a daily dosage of 3.3 mg/kg BW). Mice were food restricted before the behavioural tests of motivation and operant learning. Hedonic preference test was conducted in sated as well as food-deprived state. Tissues were collected after sacrifice and stored until further analysis (see main text).

Behavioural tests were started after mice had received PIO for 6 weeks. Testing was performed during the active (dark) phase of the day, after appropriate pre-handling (3-4 consecutive days) and habituation to the test set-ups.

Test of motivation

The procedures followed (food restriction, testing conditions and evaluation of data) were identical to those described in Chapter 3 of this thesis, according to Harb *et al.,*(2014). The tests were conducted during the 8th week of the experiment while mice were maintained on standard diet (CON) or on a diet containing PIO.

Operant conditioning (instrumental behaviour)

Testing was performed during weeks 9-10 of the experiment during which time mice were maintained on standard diet (CON) or on a diet containing PIO. Test conditions were as described in Chapter 3 (also see Horner *et al*., 2013; Harb *et al*., 2014). Briefly, mice were presented with 20 light stimuli at the centre of the touchscreen at a variable interval of 10-40 sec. After animals nose-poke the touchscreen the liquid food reward (15 µl condensed milk with 14% sugar) was delivered at the opposite site. The test consisted of one daily session which lasted a maximum of 45 min or until mice reached the criterion (finish 20 trials in less than 20 min on at least 3 consecutive days). Animals that have not reached the criterion until the 7th day of testing were excluded from statistical analysis. The following parameters were analyzed to determine instrumental learning ability of each animal: 1) trial completed/session, 2) time to complete session, 3) stimulus touches/min, and 4) activity (beam breaks/min).

Sucrose/hedonic preference test

A preference test for taste/hedonia and energy was conducted, in which animals were given the choice between water (no taste, no energy), 3.5% sucrose (taste and energy) and 0.4% saccharin (taste but no energy). Solutions were diluted in tap water. A concentration of 0.4% saccharin has been shown to be the most preferred and should also be iso-hedonic with 3.5% sucrose (Young and Madsen, 1963; Beeler *et al.*, 2012). Animals had *ad libitum* access to water and food and the consumption of solutions was measured at 3h, 6h and 24h. In order to determine whether the preference of animals is altered by their energetic needs this test was also performed in starved state, where they had *ad libitum*

access to the three solutions (water/sucrose/saccharin) but not to their food. Consumption of each fluid was assessed gravimetrically at 3 h, 6 h and 24 h here as well. Preference for each solution was calculated as follows:

$$Preference\ (\%) = \frac{Consumption\ water\ or\ sucrose\ or\ saccharin}{Total\ fluid\ consumption} * 100$$

Tissue collection

Animals were sacrificed (cervical dislocation) and the prefrontal cortex, hypothalamus and hippocampus were dissected and snap-frozen in isopentane (2-methylbutane, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Dissected brain areas were stored at -80°C until further analysis.

Statistical analysis

Statistical differences between groups were evaluated using Prism 6 software (GraphPad, San Diego, CA). After confirming for normal distribution of data, data from pairs of groups was tested by Student's t-test. To compare groups (more than two parameters), a two factor analysis of variance (2-ANOVA), followed by Sidak's multiple comparison (post hoc) tests, was used. The level of significance was set at $p < 0.05$ and numerical data are shown as mean +SEM.

4.3 Results

Pioglitazone increases body weight

Pioglitazone (PIO) significantly increased body weight (BW) (**Figure 4.1A;** $p < 0.05$). As shown in **Figure 4.1B** the absolute change in BW was also greater in the PIO-treated *vs.* control (CON) mice ($p < 0.05$) during the first 6 weeks of PIO treatment. Animals were maintained on normal chow (CON) or PIO for a further 6 weeks during which time they underwent behavioural testing, as described below; their BW at the end of the study was depicted in **Figure 4.1C.** Note, that as compared to the data shown in **Figure 3.3A** for similarly-aged mice, the animals in this experiment had starting BW that were about 10% lower than in the previous experiment, and that both, CON and PIO-treated animals showed smaller weight gains during the course of the study.

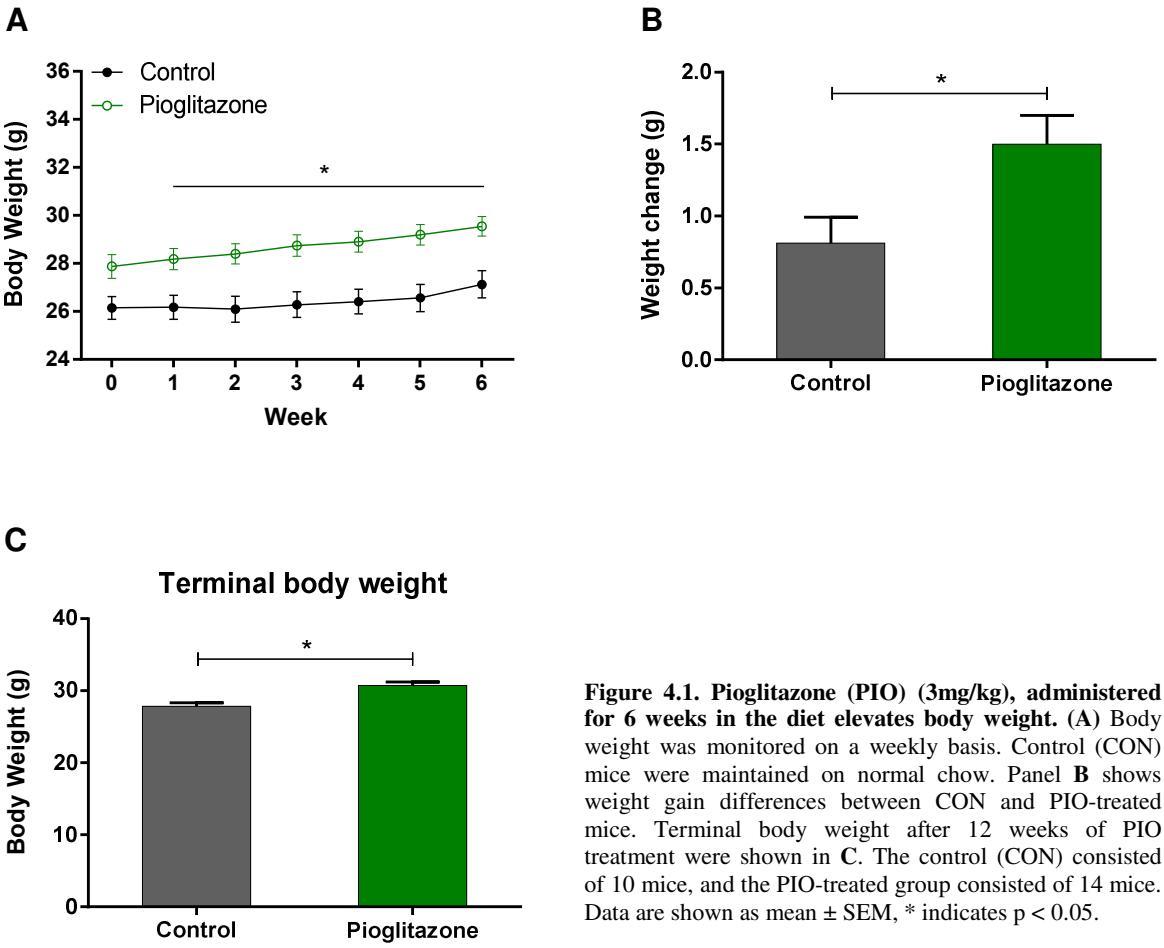


Figure 4.1. Pioglitazone (PIO) (3mg/kg), administered for 6 weeks in the diet elevates body weight. (A) Body weight was monitored on a weekly basis. Control (CON) mice were maintained on normal chow. Panel **B** shows weight gain differences between CON and PIO-treated mice. Terminal body weight after 12 weeks of PIO treatment were shown in **C**. The control (CON) consisted of 10 mice, and the PIO-treated group consisted of 14 mice. Data are shown as mean ± SEM, * indicates $p < 0.05$.

Influence of pioglitazone on motivation and appetitive learning

Hinde (1966) defined motivation as "an internal process that modifies an organism's responsiveness to a certain class of external stimuli", but this definition is still subject to much discussion despite its central place in behavioural neuroscience (Berridge, 2004). In the first set of experiments to test "pure motivation", mice received 15 presentations of a liquid food (sweetend milk) reward without having to "work" for it; each retrieval was "rewarded" with a further delivery of the reward. Starting 1 week before testing, and on all 3 test days, mice were subjected to a food restriction regimen so that their body weights throughout the testing period were 10-15% lower than during the pre-test period.

As shown in **Figure 4.2A**, both CON and PIO-treated mice displayed similar levels of locomotor activity during all test sessions. This result indicates that none of the other behavioural measures could have been confounded by differences in locomotor/exploratory behaviour. This is particularly important because the group treated with PIO had significantly higher BW at the time of testing (6 weeks after initiation of the pharmacological manipulation).

Although PIO-exposed mice took significantly longer ($p < 0.05$) to initiate "reward seeking" during the first session (reward retrieval latency, **Figure 4.2B**; time effect: $F_{2,40} = 42.16$, $p < 0.0001$; treatment effect: $F_{1,20} = 5.516$, $p = 0.0292$), neither of the test groups (CON and PIO-treated) differed on this parameter in subsequent test sessions. As similar pattern of response was shown by CON and PIO-treated mice when the time taken to complete each session was monitored (**Figure 4.2C**); notably, all animals showed a time-dependent increase in the speed of completing reward retrieval (Time effect: $F_{2,40} = 41.03$, $p < 0.0001$). Further, both groups of mice displayed a similar number of entries into the food tray on all occasions (**Figure 4.2D**).

As noted by Berridge (2012), Philip Teitelbaum suggested that "real motivation" can be recognized as "the capacity to motivate flexible instrumental behaviour". Teitelbaum posited that motivation can only be proven by the individual's ability to "learn a new operant response to gain a goal". Similar operant responses (also known as instrumental responses), where mice were required to learn to expend effort to obtain a food reward, were used here to assess the influence of PIO on motivation.

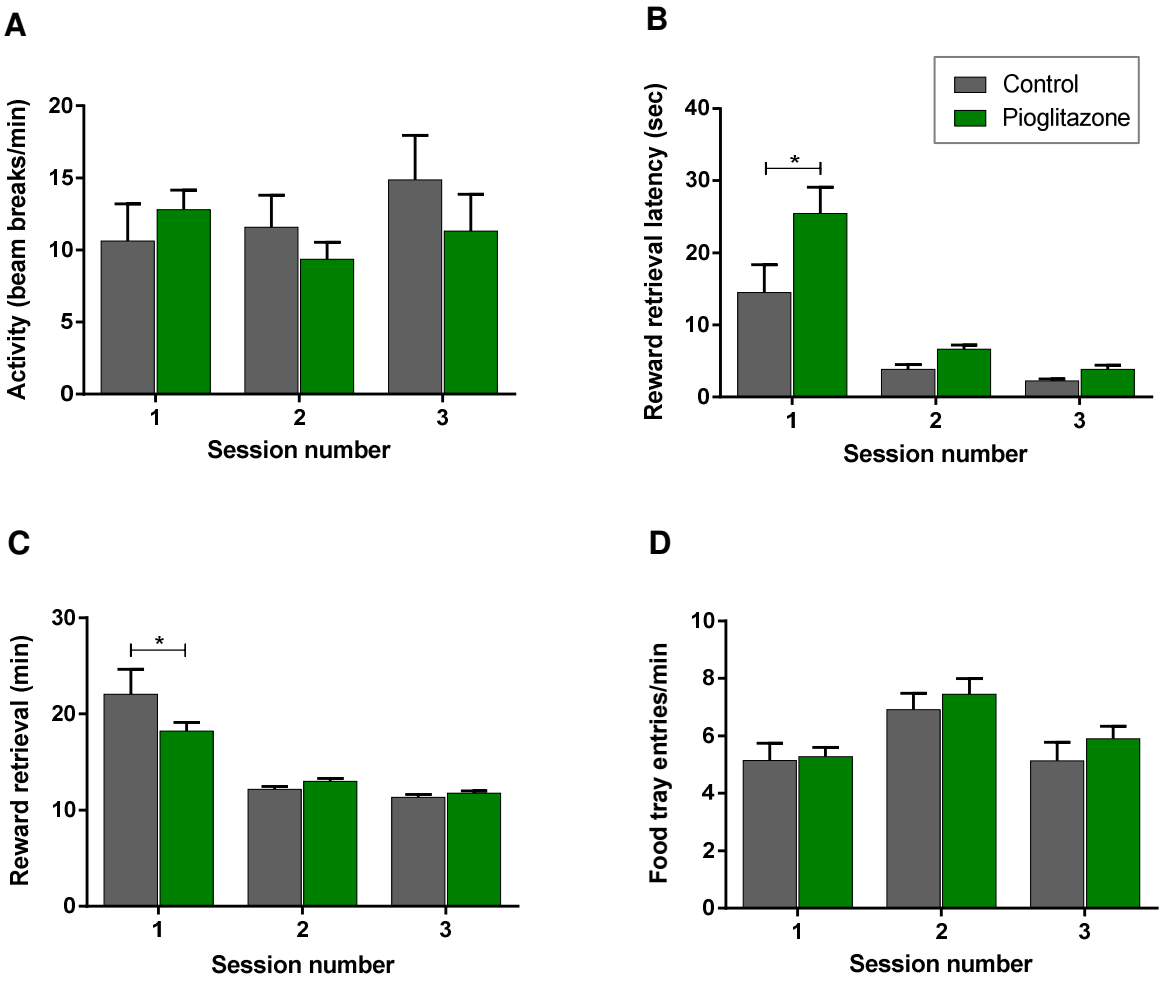


Figure 4.2. Pioglitazone does not affect motivation for a food reward. Mice received 15 liquid food reward (condensed milk with 14% sugar) presentations at a variable interval of 10-40 sec. **(A)** Activity (beam breaks/min), **(B)** Reward retrieval latency (sec), **(C)** Time to retrieve reward (min) and **(D)** Food tray entries/min were analyzed in CON (n=9) and PIO (n=13) groups of mice, that have been food restricted. The test was carried out over 3 days. Data are shown as mean ± SEM, * indicates $p < 0.05$.

Specifically, upon receiving a light cue, fasted mice had to nose-poke a touchscreen to receive a liquid food (sweetened milk) reward. Each trial consisted of 20 cues and successful completion of the trial was determined by the criterion "completion of 20 trials within 20 min on at least 3 consecutive days" over a 7-d testing period.

Activity of both, CON and PIO-treated, mice (measured by frequency of beam breaks in the touchscreen chambers) was similar during all test days (**Figure 4.3A**), with a gradual significant decrease in activity on consecutive days of testing (Time effect: $F_{6,120} = 5.802$, $p < 0.0001$). Number of trials completed (**Figure 4.3B**), time to complete an individual session (**Figure 4.3C**) and stimulus touches per minute (**Figure 4.3D**) were also similar between groups, although both parameters revealed improvements in performance over

time ($F_{6,120} = 2.619$, $p = 0.0202$, $F_{6,120} = 14.30$, $p < 0.0001$ and $F_{6,120} = 5.864$, $p < 0.0001$, respectively).

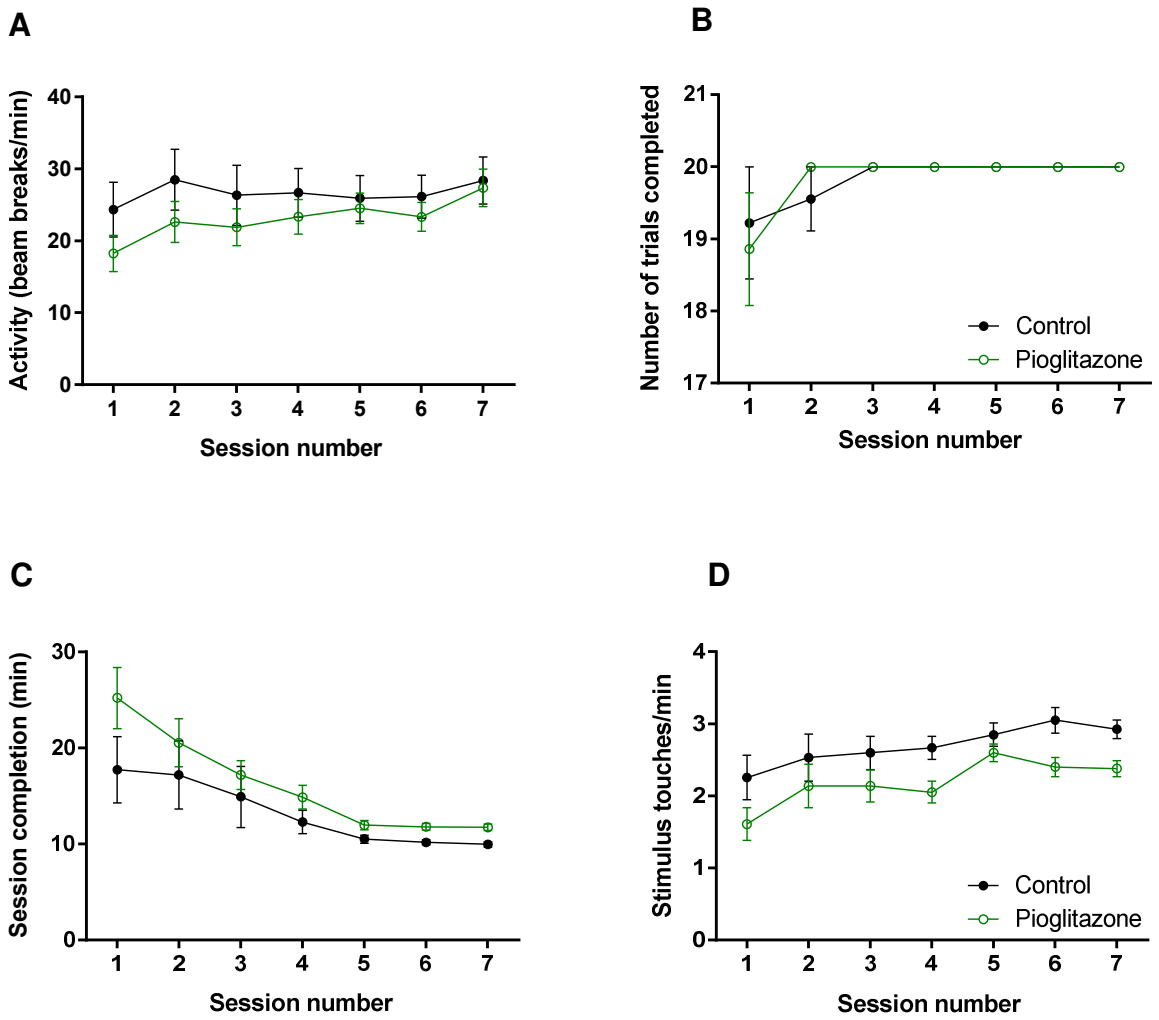


Figure 4.3. Lack of pioglitazone effects on motivational behaviour as measured by instrumental (operant) responses. Mice received 20 light stimuli at the center of the touchscreen at a variable interval of 10-40 sec. Liquid food reward (condensed milk with 14% sugar) was delivered with each "nose-poke" of the touchscreen. The test was carried out on 7 consecutive days. **(A)** Activity (beam breaks/min), **(B)** Number of trials completed, **(C)** Time to finish session (min) and **(D)** Stimulus touches/min. For the test, mice were food restricted; CON (n=9), PIO (n=13) groups of mice. Data are shown as mean ± SEM.

Taken together, the above sets of results indicate that PIO does not have the expected effect on motivation (increased) to eat, a prediction based on the observations in this chapter (and Chapter 3) that PIO treatment causes weight gain. Further, the experiments done here indicate that PIO does not interfere with an important cognitive domain, learning, and one that not only drives operant behaviour but also one that is, reciprocally, influenced by motivational state.

Body weight as a modifier of motivational state

It was concluded in Chapter 3 that PIO reduces motivation for an appetitive (food) reward) (**Figure 3.6 B-D**), a conclusion that could also be drawn from the two tests of motivation conducted in this chapter (**Figure 4.2** and **Figure 4.3**). However, the BW of mice used in Chapter 3 differed significantly ($p < 0.05$) from those used in the current experiment (**Figure 4.4A** and **B**), even though both sets of animals were of the same age and received the same dose of PIO in the food (3.3 mg/kg). This difference would appear to be important, at least in determining the degree of motivation to eat, as can be seen from **Figure 4.5**, where the data shown in Chapter 3 (Batch 1) and in the present chapter (Batch 2) are compared.

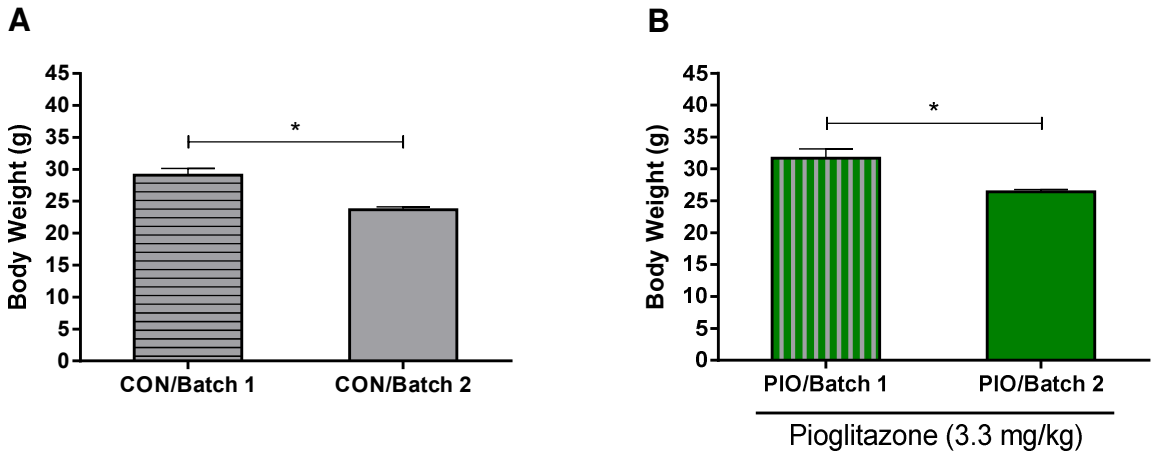


Figure 4.4. Body weights (BW) of two batches of CON and PIO-treated mice. The data derives from mice of identical ages, handling and treatment protocols; there was an interval of 9 months between the first (Batch 1, see Chapter 3) and second (Batch 2, this Chapter) experiments. Body weight change in (**A**) CON in Batch 1 (n=15) *vs*. Batch 2 (n=9) and (**B**) PIO-treated animals in Batch 1 (n=11) and Batch 2 (n=13). Data are shown as mean ± SEM, * indicates $p < 0.05$.

Scrutiny of the data obtained during all sessions in the test of "pure" motivation (non-operant behaviour), shows that, reward retrieval latency was increased (**Figure 4.5B,** *right hand panel*) ($p < 0.05$), and the number of entries into the food tray reduced, in the heavier, pioglitazone-treated mice (**Figure 4.5C,** *right hand panel*) ($p < 0.05$) from Batch 1, i.e. a higher body mass (most likely reflecting greater energy reserves) reduces motivation to collect a food reward. Evidence that higher energy depots (rather than PIO itself) are responsible for the reduced motivation for a food reward is given by the comparison between CON groups in both experiments, where animals from Batch 1 with higher BW (**Figure 4.4A**) show a significantly higher ($p < 0.05$) retrieval latency (**Figure**

4.5B, *left hand panel*) in the first session and less ($p < 0.05$) food tray entries in sessions 1 and 2 (**Figure 4.5C,** *left hand panel*). Importantly, BW did not have any effect on the levels of activity displayed by CON and PIO-treated mice, and the two batches did not differ on this measure (**Figure 4.5A**).

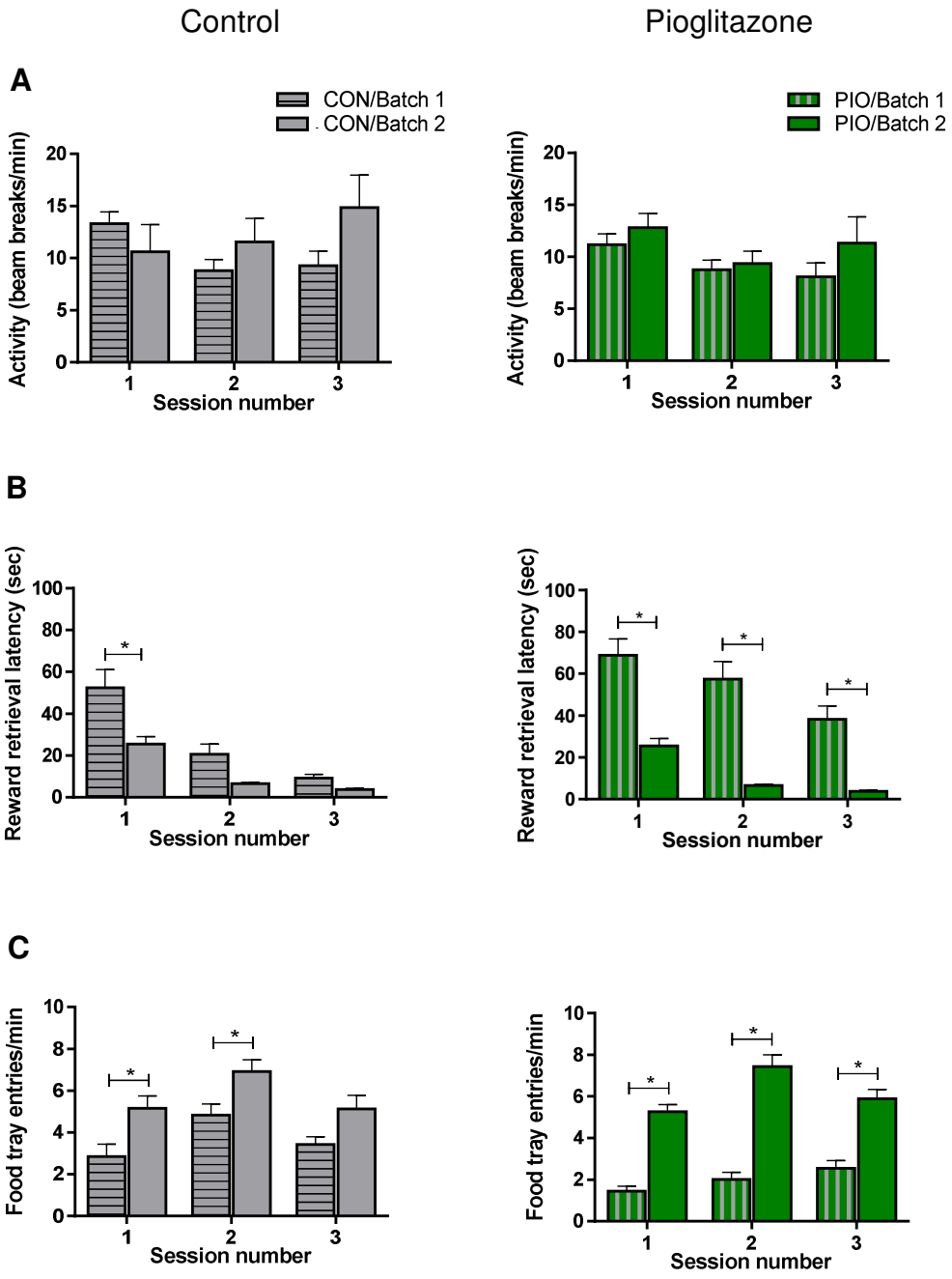


Figure 4.5. Body weight differences between Batch 1 and Batch 2 mice *(see Fig. 4.4 A, B)* **are reflected in a test of "pure motivational behaviour".** Comparison of motivation between CON (***left***) in Batch 1(hatched bars, n=15) *vs*. Batch 2 (solid bars, n=9) and PIO-treated animals (***right***) in Batch 1 (hatched bars, n=11) and Batch 2 (solid bars, n=13). Batches were tested under identical conditions, including; food-restriction as described, and 15 liquid food reward (condensed milk with 14% sugar) presentations at a variable interval of 10-40 s in each session (**A)** Activity (beam breaks/min), (**B)** Latency to retrieve the food reward (sec); (**C)** Food tray entries per minute. Data are shown as mean ± SEM, * indicates $p < 0.05$.

Similar results were observed in the operant conditioning test of motivational behaviour, with animals weighing more (Batch 1) displaying an impaired appetitive learning behaviour (**Figure 4.6**). In particular, PIO-mice weighing more (Batch 1) needed significantly more time ($p < 0.05$) to finish most of the sessions (**Figure 4.6A**, *right hand panel*) and showed also reduced stimulus touches per minute (**Figure 4.6B,** *right hand panel*) ($p < 0.05$), whereas CON mice from both batches (**Figure 4.6A** and **B**, *left hand panel*), showed similar results (albeit only during initial session/days of the task), indicating that they learnt the task faster compared to the PIO mice which displayed higher BW.

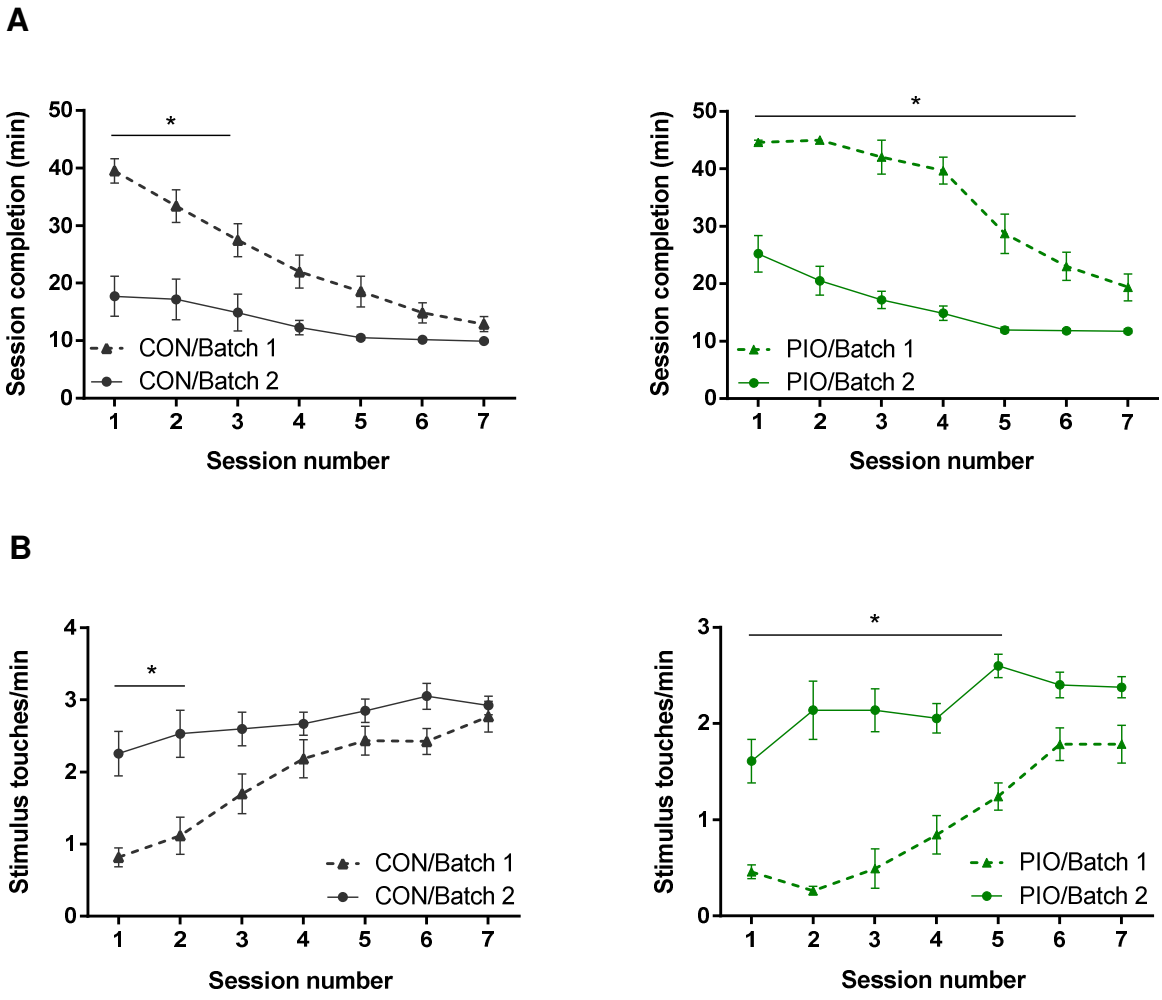


Figure 4.6. Pioglitazone reduces operant learning behaviour in mice displaying higher body weight. Comparison of operant learning between CON (**left**) in Batch 1(n=15) *vs*. Batch 2 (n=9) and PIO-treated animals (**right**) in Batch 1 (n=9) and Batch 2 (n=13). Mice received 20 light stimuli at the center of the touchscreen at a variable interval of 10-40 sec. Liquid food reward (condensed milk with 14% sugar) was delivered with each "nose-poke" of the touchscreen. **(A)** Time to finish session (min) and **(B)** Stimulus touches/min. For the test, mice were food restricted. Data are shown as mean ± SEM, * indicates $p < 0.05$.

In summary, our results support the hypothesis that motivation and thus also appetitive learning in PIO-treated mice are decreased as a consequence of an overweight state.

Effect of pioglitazone treatment on hedonic preference

Organisms develop motivational behaviours because they anticipate, or actually receive, a reward: In many cases, pursuit of that reward can have beneficial effects, but at least in humans, the pleasure (hedonia) associated with a reward plays an important role in motivating the individual to seek the reward. Foods not only contain energy (a necessity for life) but often have properties (taste, smell, texture) that elicit feelings of pleasure. In an attempt to distinguish between how the different (hedonic and non-hedonic) components impact on our observed effects of PIO on motivation, we next devised a "hedonic preference" test in which animals had free access to drinking solutions consisting of water (tasteless, no energy), 3.5% sucrose (sweet, with 3.87 kcal/g) and 0.4% saccharin (sweet, no energy). The concentrations of sucrose and saccharin were chosen on the basis of their iso-hedonic properties (Young and Madsen, 1963; Beeler *et al*., 2012). The test was carried out in mice that were sated (food available *ad libitum)* or fasted in order to control for energetic state.

Total fluid (water, sucrose and saccharine) consumption was greater in fasted mice, but 24 h consumption was significantly reduced in the fasted animals that had received PIO **(Figure 4.7A** and **B).**

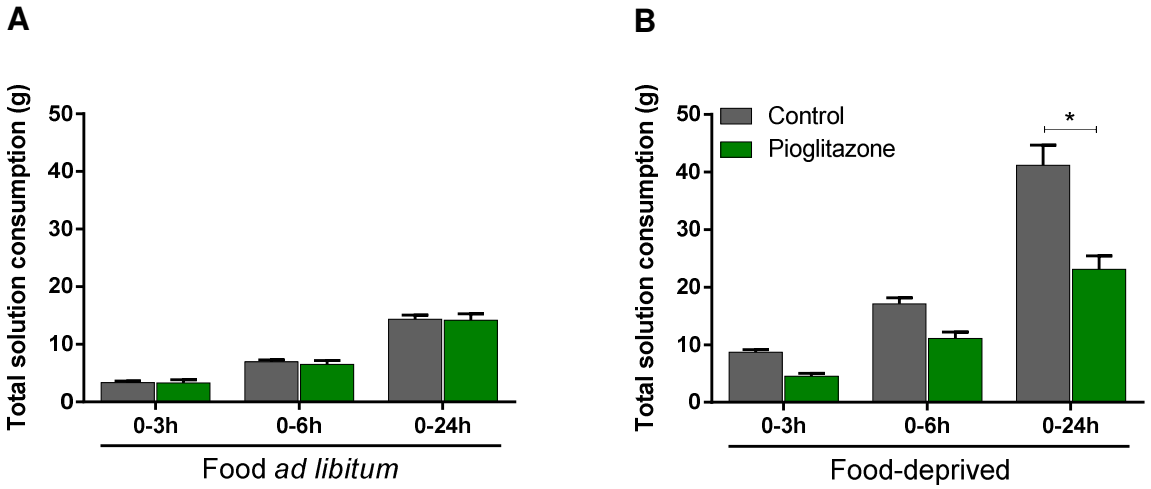


Figure 4.7. Pioglitazone decreases 24h total consumption of solution (g) in food-deprived animals during preference test. Animals were given the choice between water, sucrose or saccharin (*ad libitum*). Total consumption of solutions was measured at 3h, 6h and 24h when mice had **A)** *ad libitum* access to food **or** were **B)** Food-deprived. Control (CON) group consisted of 10 mice and pioglitazone (PIO) group of 14 mice. Data are shown as mean ± SEM, * indicates $p < 0.05$.

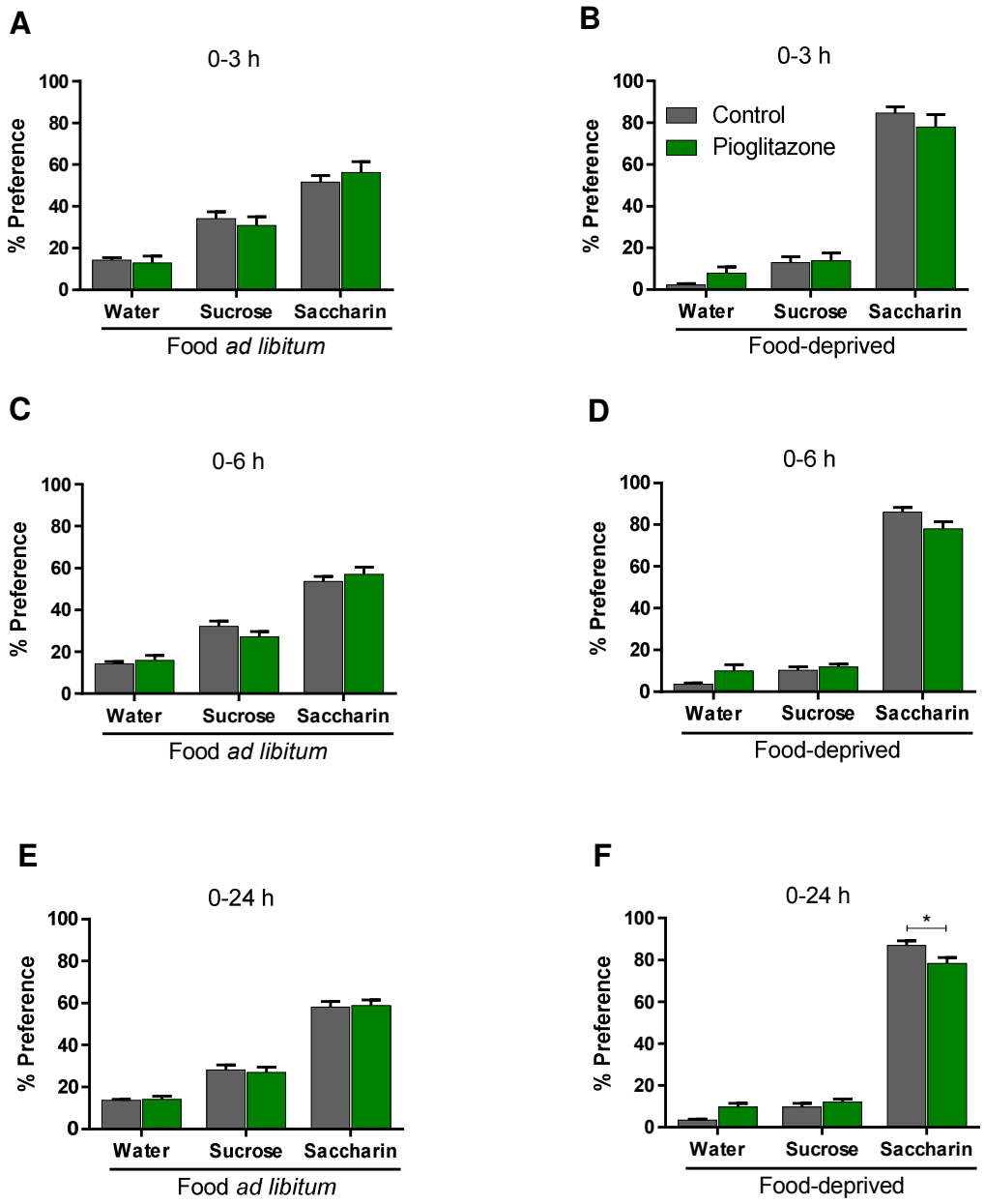


Figure 4.8. Pioglitazone decreases saccharin consumption in food-deprived animals after 24h. Relative preference [(Volume of individual solution drunk /volume of all solutions consumed)*100] between water, sucrose and saccharin was measured in CON (n=10) and PIO (n=14) groups. Relative consumption of solutions (% Preference) after 3 hours in **(A)** animals with *ad libitum* access to food and **(B)** food-deprived animals. The 0- 6 hour time-point is shown in **(C)** animals with *ad libitum* access to food and **(D)** food-deprived animals. Relative consumption after 24 hours in **(E)** animals with *ad libitum* access to food and **(F)** food-deprived animals. Data are presented as mean ± SEM, * indicates $p < 0.05$.

The reduced fluid needs of PIO-treated animals, at least in the fasted state, may relate to the fact that TZDs are known to cause fluid-retention in humans (Basu *et al*., 2006; Bełtowski et al., 2013; Soccio *et al*., 2014). In the present study, water intake was similar in CON and PIO-treated mice, although there was a tendency for PIO-treated animals that

were food-deprived to consume more water when compared to CON (**Figure 4.8 B, D** and **F**). The data shown in **Figure 4.8** illustrate the striking preference of both, sated (**Figure 4.8,** *left hand panels*) and fasted (**Figure 4.8,** *right hand panels*) mice for saccharin. Interestingly, PIO-treated animals consume less ($p < 0.05$) saccharin when compared to CON (**Figure 4.8F,** *right hand panel*) in food-deprived state.

4.4 Discussion

The question addressed in this chapter concerned the possible behavioural mechanisms that might underlie the increase in body weight (BW) seen in mice fed a diet containing pioglitazone (PIO) (this Chapter and Chapter 3 of this thesis). Since PIO is an insulin-sensitizing drug, probably involving a resetting of mechanisms that regulate energy homeostasis, we here specifically sought to examine whether an undesired (and confounding) side effect of the drug might be due to the known effect of TZDs to increase BW. Acquisition of calories and other nutrients is essential for life and a behaviour that depends strongly upon motivation - the specific behaviour examined in the above set of experiments. However, eating is also driven by the hedonic properties of food that reflect various sensory (taste, texture, smell) properties rather than the energy value of the food *per se*; the former are considered to make food (or any other pleasure-giving stimulus, e.g. alcohol, drugs of abuse, sex) "rewarding" (Berridge, 2004; Berridge and Kringelbach, 2011). Reward itself, consists of three processes: liking, wanting, and learning (Berridge and Kringelbach, 2011, 2013 and 2015). Motivation drives the "wanting" process, mostly at the beginning of the reward-cycle and pleasure/hedonia drives the "liking" component during the eating phase of the cycle; learning, which is substantially influenced by the nature of the reward plays an important role throughout the whole feeding process (Berridge, 2004; Berridge and Kringelbach, 2015)

Recent studies in animals have implicated PPARγ signaling in the motivation and reward pathway, especially in view of evidence that the ventral tegmental area (VTA), a source of dopamine and an important component of the reward pathway, expresses PPARγ within its rostromedial portion (de Guglielmo *et al*. 2015). Further, de Guglielmo *et al*. (2015) reported that oral PIO in rats attenuates their motivation to seek heroin in parallel with reduced extracellular levels of dopamine in the shell of the nucleus accumbens (Acb which receives projections from the VTA). A role for central PPARγ in motivational

behaviour was suggested in a different set of studies in an alcohol-preferring line of rats in which PIO was found to reduce alcohol consumption (Stopponi *et al*., 2011, 2013), an effect that was blocked by central administration of GW9662, a PPARγ antagonist (Stopponi *et al*., 2011).

In keeping with the above findings by Stopponi *et al*. (2011, 2013) and de Guglielmo *et al*. (2015) on motivation to seek heroin and alcohol, the results of the present study, in which two different tests of motivation were employed, at first glance suggest that PIO interferes with the neural mechanisms that control motivational behaviour, and specifically, the motivation to eat. This could be also be interpreted as a reflection of the fact that PIO-treated mice display increased BW (with accompanying increases in white adipose tissue mass), and therefore, greater energy depots, rather than a direct effect on the neural mechanisms that control motivational behaviour. Indeed, a comparative analysis of data obtained in Chapter 3 (Batch 1 mice) and the present Chapter (Batch 2 mice) gave credence to the latter view. For inexplicable reasons (animal ages, conditions and test conditions were identical for both batches of mice), the first set of control (CON) and PIO-treated litter mates showed higher BW than animals in Batch 2. Our inspection of the data revealed that Batch 2 animals (CON and PIO-treated) were more strongly motivated than CON and PIO-treated mice in Batch 1, leading to the conclusion that BW (energy reserves – see (Hariri and Thibault, 2010), rather than PIO *per se,* determines motivation to eat. While we are currently seeking mathematical support for this conclusion, we would tentatively suggest that energy status is an important factor in calibrating the drive to eat. Nevertheless, the aforementioned reports that PIO can directly influence motivational state in general (cf. Stopponi *et al*. 2011, 2013; de Guglielmo *et al*. 2015), through the mediation of central PPARγ, cannot be dismissed and deserve further in-depth investigation.

Additional factors must also be taken into account when considering our results on PIO-induced BW gain. For example, overweight and obesity have been associated with cognitive impairment, specifically in learning and memory (Farr *et al*., 2008; Smith *et al*., 2011; Heyward *et al*., 2012; Valladolid-Acebes *et al*., 2013; Harb and Almeida, 2014; Nguyen *et al*., 2014). Harb & Almeida (2014) also showed that PIO-treated overweight animals are less motivated to consume a food reward, similar to the findings of the present work (including the comparison between Batch 1 and Batch 2 mice).

As noted above, hedonic factors play an important role in modifying motivated behaviours such as eating. In this respect, it should be mentioned that, at least in humans, pleasure signals work to override homeostatic controls on caloric intake, resulting in overweight and obesity (Berridge, 2004; Lowe and Butryn, 2007; Beeler *et al*., 2012; Berridge and Kringelbach, 2013, 2015). While animals may also display a similar competition between hedonic and homeostatic controls, our group previously suggested that animals which, unlike humans, do not have easy access to an abundant (and rich variety) supply of hedonically-charged foods, can better use homeostatic mechanisms to titrate their intake of calories to match their consumption of food with their energetic needs, than humans (Harb and Almeida, 2014).

To further explore the idea of interactions between hedonic drive and energetic status and their modulation by PIO, we here devised a test of hedonic preference in which sated mice (with *ad libitum* access to food) were allowed to choose to drink from bottles containing water (no taste, no calories), 3.5% sucrose (sweet, caloric), or 0.4% saccharin (sweet, non-caloric); note that the sucrose and saccharin solutions are reportedly iso-hedonic (Young and Madsen, 1963; Beeler *et al*., 2012). The test was subsequently repeated in the same set of animals that had been fasted overnight (and food-restricted during testing), so as to examine the extent to which energetic needs influenced preference for the different drinking solutions. In general, fasting increased fluid intake, whereas PIO-treated animals showed lower solution consumption compared to CON. Whether this can be explained by previous reports that TZDs cause water retention (Guan *et al*., 2005; Basu *et al*., 2006; Bełtowski *et al*., 2013; Soccio *et al*., 2014) is not clear at present.

A striking finding was that both, fasted (presumably in negative energy balance) and sated mice, consumed the sweet, non-energetic saccharin solution rather than sucrose (and water). At least initially, this is explicable by the intense sweetness of saccharin (*versus* sucrose and tasteless water) and possibly the anticipation of energy input (sweetness-energy association seems to be inherently learnt in many species); however, and especially because carbohydrates deliver energy rapidly and because energy can be rapidly sensed by the hypothalamus, one would expect that the fasted mice would eventually switch to drinking sucrose to replenish their energy reserves. Such a switch was not apparent, and sated/fasted CON and PIO-treated mice behaved similarly except that over the full 24 h cycle monitored, the fasted PIO-exposed mice drank significantly

less saccharin than their CON counterparts did. On the other hand, it is tempting to suggest that the greater BW and energy depots (as well improved insulin sensitivity) in the PIO-treated mice leads to their faster loss of motivation to seek potential sources of energy. This interpretation matches our previous report (Chapter 3) that "pure motivation" and appetitive learning are reduced in mice rendered overweight by PIO treatment. Moreover, since the hedonic properties of a reward promote motivation to acquire that reward, it is proposed that PIO can act to reduce the hedonic valence of an otherwise highly-palatable stimulus; in this way, PIO would help restore to the balance between hedonic and homeostatic pressures to eat. These suggestions clearly deserve further investigation as they might contribute to our understanding of how to curtail hedonic feeding before it leads to overweight and obesity, eventually raising the risk for neuropsychiatric disorders, including Alzheimer's disease.

CHAPTER 5

General discussion

The overarching goal of the present research in mice was to attempt to obtain a stronger basis for the claim that activated peroxisome proliferator-activated receptor (PPAR) of the γ type (PPARγ) is important for optimal brain function, in particular, with respect the maintenance and/or restoration of cognitive function in Alzheimer's disease (AD) (Heneka and Landreth, 2007; Zolezzi *et al*., 2014; Pérez and Quintanilla, 2015). One question of particular interest was whether the central actions ascribed to PPARγ agonists occur directly or represent effects that are secondary to their actions in the periphery. While these aims were only partially fulfilled, for reasons discussed below, the work generated a number of novel insights into PPARγ-regulated metabolic and behavioural interactions that may ultimately impact on our understanding of how metabolic dysfunction may contribute to AD.

As PPARγ, a master regulator of lipid and glucose metabolism (Lehrke and Lazar, 2005; Cho *et al*., 2008; Tontonoz and Spiegelman, 2008; Wahli and Michalik, 2012) has been linked to cognition and stress physiology (García-Bueno *et al*., 2005a, b; García-Bueno *et al*., 2008a, b; Ryan *et al*., 2012), our hypothesis was that this receptor may link peripheral metabolism with brain health and disease. Thus, we sought to examine the actions of pioglitazone, a PPARγ agonist, during stress and its ability to reverse stress-induced dysfunction over time, including metabolic and cognitive disturbances (**Chapter 3**). One important question to address was if TZD actions on cognition are direct on the brain (central) or due to their peripheral improvement on insulin sensitivity. This question became interesting, because to the limited and contradictive information about PPARγ expression in mouse brain. Although, the presence of PPARγ has been thoroughly reported in rat brain and spinal cord (Braissant *et al*., 1996; Cullingford *et al*., 1998; Moreno *et al*., 2004; Cimini *et al*., 2005; Inestrosa *et al*., 2005; Sarruf *et al*., 2009), the information about the presence and role of PPARγ in the mouse brain is still debated. Additionally, PPARγ has been implicated in the regulation of hypothalamic circuits that control feeding and energy metabolism (Diano *et al*., 2011; Lu *et al*., 2011; Ryan *et al*., 2011; Garretson *et al*., 2015; Liu *et al*., 2015). In light of the importance to distinguish between central and peripheral actions of PPARγ, we first tried to examine the presence of PPARγ in areas involved in cognition, neuroendocrine function and energy balance. In order to understand specificity and central regulation of PPARγ we sought to analyze its distribution on brains of mice that received high fat diet (**Chapter 2**). Furthermore, the influence of pioglitazone treatment on motivation and appetitive learning revealed in this

study triggered our interest to investigate the involvement of PPARγ activation in motivation, reward and hedonia (**Chapter 4**), as it has been already reported to reduce opioid and alcohol consumption.

Cognitive impairments generally increase over the lifespan; indeed, age is the most important risk for developing AD and other dementias (LaFerla and Oddo, 2005; Mu and Gage, 2011). A small number of AD cases result from genetic predisposition that can be ascribed to mutations in the amyloid precursor protein (APP) gene or in the presenilin 1 and 2 genes (LaFerla and Oddo, 2005; Merlo *et al*., 2010; Holtzman *et al*., 2011) causing early-onset AD. In contrast, the majority of AD cases are the late-onset form of AD, driven by a diverse set of triggers. One of the most important risks for late-onset AD is the expression of the ε4 allele of the apolipoprotein E (ApoE) gene; the ApoE protein normally contributes to lipid homeostasis by facilitating lipid transport between cells and tissues but also to the clearance of toxic amyloid peptides (Roses, 1996; Huang *et al*., 2004; Liu *et al*., 2013). In recent years, studies in humans and animals have pointed to the possible importance of stress and stress-related disorders such as depression (Sotiropoulos *et al*., 2008b; Patchev *et al*., 2014; Vyas *et al*., 2016; Kaup *et al*., 2016; Mirza *et al*., 2016) as well as obesity and type 2 diabetes (T2D) in making individuals vulnerable to AD pathology (Rasgon and Kenna, 2005; Winocur and Greenwood, 2005; Craft, 2007; Farr *et al*., 2008; de La Monte, 2009; Merlo *et al*., 2010; Smith *et al*., 2011; Luchsinger, 2012; Nguyen *et al*., 2014; Pérez and Quintanilla, 2015; Heneka *et al*., 2015b). The initial idea to test PPARγ agonists in AD therapeutics was probably triggered by the causal link between T2D (and other metabolic disorders) and AD, because activation of PPARγ is an effective way to restore insulin sensitivity. The thiazolidinedione (TZD) class of agonists, best represented by rosiglitazone and pioglitazone, have proven to be very effective drugs for the clinical management of T2D (Hofmann and Colca, 1992; Nolan *et al*., 1994; Lehmann *et al*., 1995; Willson *et al*., 1996; Willson *et al*., 2000; Berger and Moller, 2002; Sauer, 2015), although their use has been somewhat limited by the fact that they have been linked to cardiovascular complications and bladder cancer in a small number of cases (Cariou *et al*., 2012; Ahmadian *et al*., 2013; Soccio *et al*., 2014; Sauer *et al*., 2015).

Does the mouse brain express PPARγ?

As already mentioned, although PPARγ activation by TZD therapy reportedly ameliorates some of the cognitive deficits observed in human AD patients and animal models of AD, it remains unclear as to whether the effects reflect primary, direct effects on the brain or are consequences of their ability to improve insulin sensitivity and metabolism in general. The ability to detect PPARγ in the brain was considered a critical piece of evidence needed to answer this question. Accordingly, biochemical and morphochemical methods were applied to examine PPARγ expression in the brains of mice that were exposed to physiological stimuli in order to verify that the detected entities are regulatable in a predictable fashion (see **Chapter 2**). This analysis, based on radioactive *in situ* hybridization histochemistry (ISHH) revealed very low levels of PPARγ mRNA in the central nervous system (CNS), allowing only a qualitative assessment of the results. Notably, highest levels of receptor mRNA were found in the ventromedial hypothalamus (VMH) and hippocampus of mice maintained on a high fat diet (HFD). The VMH plays an important role in the regulation of feeding behaviour and directly responds to glucose and a variety of other feeding- and energy intake-regulatory signals (King, 2006), whereas the hippocampus is important for cognitive functions such as learning and memory, the control of mood and emotions, as well as the regulation of the neuroendocrine response to stress (Reul and de Kloet, 1985; Herman *et al*., 1996; Wall and Messier, 2001; Mizoguchi *et al*., 2003; Samson and Barnes, 2013; Strange *et al*., 2014). It should be mentioned that the literature contains equivocal reports regarding the presence of PPARγ mRNA in specific areas of the brain and their regulation by specific stimuli. For example, whereas Diano *et al.* (2011) found that HFD upregulates PPARγ mRNA expression (and peroxisome numbers) in the hypothalamus, Liu *et al.* (2015) failed to see such an effect but rather reported that fasting specifically increases PPARγ mRNA in one hypothalamic nucleus, the suprachiasmatic nucleus (SCh); the latter study employed both chromogenic ISHH and qPCR in laser-capture microdissected samples.

In this work, attempts to visualize PPARγ signal using immunodetection (immunohistochemistry and immunoblotting) were generally unsuccessful. Interestingly, however, PPARγ protein levels in hippocampus were found to be increased, albeit not significantly, when mice received pioglitazone (PIO), a TZD agonist of PPARγ. In genral, weak PPARγ immunoreactivity was observed in brain areas involved in cognitive processes such as learning and memory, and in the control of motivation, mood and

emotion (cortex, hippocampus), as well as the regulation of the neuroendocrine and behavioural response to stress (cortex, hippocampus and hypothalamus) and feeding behaviour (hypothalamus). These findings tentatively confirm the existence of physiologically-relevant PPARγ in the brain, a view supported by several other reports in the literature. Potential (technical) reasons for why demonstration of immunoreactive PPARγ in the mouse brain becomes a challenge, and often leads to differing claims (cf. (Braissant *et al*., 1996; Cullingford *et al*., 1998; Moreno *et al*., 2004; Cimini *et al*., 2005; Inestrosa *et al*., 2005; Sarruf *et al*., 2009; Diano *et al*., 2011; Lu *et al*., 2011; Liu *et al*., 2015), were discussed extensively in **Chapter 2**. It is however important to note that our group recently detected both, PPARγ mRNA and protein in primary hippocampal cultures obtained from 4 days old mice (S. Moosecker, *unpublished data)*; briefly, PPARγ was seen to be expressed by neurons, and at much lower levels, by astrocytes and oligodendrocytes. These findings are partly consistent with earlier work describing a gradual reduction in PPARγ mRNA expression during embryonic development of the rat brain (mRNA encoding the receptor becomes undetectable by embryonic day E18.5) (Braissant and Wahli, 1998).

The present mRNA and protein expression studies suggesting that PPARγ is absent from most but a few brain nuclei need to be reconciled with previous pharmacological data indicating the presence of functional PPARγ in brain. For example, Ryan *et al*. (2011) and Garretson *et al*. (2015) reported that central (intracerebroventricular, ICV) injections of rosiglitazone (a TZD) increases feeding in rats, and hamsters, respectively. In other studies, central administration of rosiglitazone was also shown to reverse memory deficits in diabetic mice (Kariharan *et al*., 2015) and when administered directly into the right dentate gyrus of rats treated with Aβ42 (Xu *et al*., 2014), while Denner *et al.* (2012) demonstrated that the improvement of hippocampus-dependent cognition in an AD transgenic mouse by dietary rosiglitazone can be blocked by central (intracerebroventricular, ICV) injections of the PPARγ antagonist GW9662. In addition, dietary rosiglitazone was shown to improve synaptic activity and neuronal firing properties in the hippocampus of APP-overexpressing mice (Nenov *et al*., 2014, 2015). Thus, there is a rather strong body of evidence that indicates the presence, regulation and function of PPARγ in the brain; on the other hand, data on the visualization of central PPARγ using immunodetection methods is weak. Further, apart from the studies in which direct application of PPARγ agonists or antagonists to the brain resulted in measurable

changes in neuronal function or behaviour, the available data on the central effects of PPARγ ligands does not necessarily exclude actions that occur secondary to effects on peripheral metabolism. Clearly, further effort and improvements in the detection of central PPARγ is needed, especially if PPAR-mechanisms are to be exploited for ameliorating disorders of the brain such as Alzheimer's disease.

The adverse effects of chronic stress or exposure to exogenous glucocorticoids (GCs) on health, range from metabolic disorders (e.g. obesity, type 2 diabetes) to impairments of mood and cognition (see Sapolsky, 2000; Rasgon and Kenna, 2005; Cerqueira *et al*., 2005, 2007; Depke *et al*., 2008; Sotiropoulos *et al*., 2008a; Catania *et al*., 2009; Chrousos, 2009; Sotiropoulos *et al*., 2011; Rostamkhani *et al*., 2012; Sousa and Almeida, 2012; Detka *et al*., 2013; Li *et al*., 2013). Of particular interest to the present work, is the observation that hypercortisolemia is commonly found among AD patients and has been, accordingly, linked with the disease (Hartmann *et al*., 1997; Weiner *et al*., 1997; Rasmuson *et al*., 2001; Csernansky *et al*., 2006; Elgh *et al*., 2006; Sotiropoulos *et al*., 2008b). The latter view is supported by previous work from our group and others which shows that chronic stress or glucocorticoid treatment in rats and mouse models of AD stimulates the development of AD-like neuropathology (Green *et al*., 2006; Jeong *et al*., 2006; Sotiropoulos *et al*., 2008a; Catania *et al*., 2009; Sotiropoulos *et al*., 2011). It is also of interest to note that obesity and type 2 diabetes (T2D) appear to increase the risk for the development of cognitive impairment (including Alzheimer's disease) (Farr *et al*., 2008; Merlo *et al*., 2010; Smith *et al*., 2011; Heyward *et al*., 2012; Luchsinger, 2012; Valladolid-Acebes *et al*., 2013; Nguyen *et al*., 2014; Pérez and Quintanilla, 2015; Heneka *et al*., 2015b), possibly due to the bidirectional link between stress/hypercortisolemia and insulin resistance in humans and animals (Rasgon and Kenna, 2005; Zardooz *et al*., 2006; Depke *et al*., 2008; Rostamkhani *et al*., 2012; Ghalami *et al*., 2013; Li *et al*., 2013; Detka *et al*., 2013; Hwang and Weiss, 2014).

Attempts to untangle the relationships between stress and metabolism and their regulation by PPARγ

One of the present study's objectives was to investigate the link between stress, metabolism, and cognition and their modulation by activation of PPARγ with pioglitazone (PIO). Several published studies suggest that PPARγ signaling plays a role

in the regulation of the physiological response [elevated glucocorticoid (GC) secretion and increased levels of pro-inflammatory cytokine peptides] to stress (García-Bueno *et al*., 2005a, b; García-Bueno *et al*., 2008a, b; Ryan *et al*., 2012). This subject was revisited in **Chapter 3** of this thesis as a prelude to examining the modulatory role of PIO on stress-triggered alterations in metabolism and cognition in mice. The chronic unpredictable stress (CUS) paradigm used resulted in increased blood corticosterone (CORT, the predominant GC secreted by the mouse adrenal gland) levels, and, in parallel increased locomotor activity (evaluated in an open field arena) and decreased body weight (BW). Interestingly, although PIO prevented the stress-induced loss of BW, in keeping with its known actions (Lehrke and Lazar, 2005; Cariou *et al*., 2012; Soccio *et al*., 2014), the drug accentuated the stress-induced increase in CORT secretion. This latter result, which contrasts with the reported GC-dampening effect of another TZD (rosiglitazone) in rats that were only acutely restraint- stressed (Ryan *et al*., 2012), suggests that the effects of TZDs depend on the chronicity or quality of the applied stressor. On the other hand, and somewhat consistent with the previously-mentioned results reported by Ryan *et al.* (2011), PIO attenuated the response of mice to an acute stressor in non-stressed mice (at the end of the experiment). It is also interesting to note that, at the termination of the experiment, PIO treatment of the stressed (STR) mice was not associated with an increased mass of white adipose tissue mass (WAT). Unfortunately, the latter and all the other experimental data obtained in these studies, are limited by the fact that biological and behavioural assessments could not be made at the end of the CUS paradigm also for practical reasons (point at which confound-free behavioural evaluations could be made), as well as restrictions on the availability of animal housing. Nevertheless, our findings do not contradict the view that lipid and energy metabolism play an important role in stress physiology even if extricating the individual components and interactions represents a major challenge.

While stress itself acts as a catabolic stimulus, often leading to loss of BW, stress can also cause obesity (and T2D) (Asensio *et al*., 2004; Wake and Walker, 2004; Seckl and Walker, 2004; Wang, 2005) by triggering lipolysis and the synthesis of triglycerides (Dallman *et al*., 2003; Dallman, 2010; de Guia *et al*., 2015). The mechanism through which GC cause obesity may also be related to the regulation of two isoforms of the enzyme 11β-hydroxysteroid-dehydrogenase (11β-HSD1 and 11β-HSD2) which normally maintain the balance between active and inactive glucocorticoids; 11β-HSD1 converts the

inactive GC cortisone into active GC (cortisol in humans, CORT in rodents) in a variety of tissues, including adipose tissue, liver and brain, whereas 11β-HSD2, which catalyses the reverse reaction, is mainly found in the colon, kidney and salivary glands (and placenta) (Seckl and Walker, 2004; Wang, 2005). Obesity in animals and humans associate with 2-3-fold higher levels and activity of 11β-HSD1 in adipose tissue which, in turn, correlate with metabolic disturbances such as glucose intolerance and insulin resistance (Asensio *et al*., 2004; Wake and Walker, 2004; Seckl and Walker, 2004; Wang, 2005). It is therefore plausible that the higher levels of CORT found after CUS+PIO (but also basal CORT levels in PIO mice at the end of the experiment) result from their higher BW and WAT mass that perturb the 11β-HSD isoform balance. Strikingly, the temporal relationship between stress/GC levels and the development of BW/metabolic dysregulation, and the role of 11β-HSD in this relationship, remains enigmatic but possibly reflective of an underlying vicious circle. It is also interesting that Sandeep *et al*., (2004) have demonstrated the presence of 11β-HSD1 in the hippocampus, and shown that its deletion improves cognition (Sandeep *et al*., 2004). It would be interesting to know the extent to which TZDs modulate central 11β-HSD, and whether such a mechanism might explain the cognition-improving effects of TZDs, as reported by some investigators.

The previous and present work highlights an interesting, but unsolved paradox: if activation of PPARγ by TZDs induces BW gain and raises the risk for development of insulin sensitivity, how do TZDs have the potential to improve brain health, including the reversal of cognitive impairments in AD patients and animal models expressing biochemical markers and behavioural phenotype of the disease? It is important to mention, however, that this paradox may, at least partly be explained by the fact that although TZDs increase BW and total fat mass, the increase in the latter results from an increase in the number of small fat cells which have a higher potential to store lipids (with parallel reductions in circulating free fatty acid levels and improved insulin sensitivity (de Souza *et al*., 2001). Indeed, PPARγ activation by TZDs results in fatty acid uptake and storage in adipose tissue, while shifting/fluctuating the storage from liver and muscle into adipose tissue (Cariou *et al*., 2012; Ahmadian *et al*., 2013; Soccio *et al*., 2014; Sauer *et al*., 2015). Further, it should be kept in mind that, since PPARγ act as transcription factors to promote adipogenesis (Lehrke and Lazar, 2005; Cho *et al*., 2008; Tontonoz and Spiegelman, 2008; Wahli and Michalik, 2012; Ahmadian *et al*., 2013), BW gain after

TZD treatment (Lehrke and Lazar, 2005; Cariou *et al*., 2012; Soccio *et al*., 2014) is not necessarily an unexpected outcome.

In light of results obtained in the present work, it is interesting to mention that CUS has been reported to induce hyperphagia (Teegarden *et al*., 2008; Sanghez *et al*., 2013; Razzoli *et al*., 2015), even though stress, a multidimensional (quality, chronicity, intensity) stimulus, may either induce weight gain or weight loss (Razzoli and Bartolomucci, 2016), depending on the particular properties of the stimulus which, in turn, may recruit quite divergent, convergent or overlapping physiological pathways. The hyperphagia-inducing effects of stress are generally attributed to altered activity of neurons in the arcuate nucleus of the hypothalamus which produce orexigenic peptides, namely agouti-related peptide (AGRP) and neuropeptide Y (NPY) under stimulation of GC (Lu *et al*., 2002; Patterson *et al*., 2013). Another appetite-stimulating hormone, ghrelin (produced by the stomach) has also been shown to be elevated after stress (Patterson *et al*., 2013; Sominsky and Spencer, 2014; Razzoli and Bartolomucci, 2016) while stress triggers gluconeogenesis and may, eventually, lead to impaired glucose metabolism and insulin resistance (Zardooz *et al*., 2006; Depke *et al*., 2008; Rostamkhani *et al*., 2012; Ghalami *et al*., 2013; Li *et al*., 2013; Rasgon and Kenna, 2005; Hwang and Weiss, 2014; Detka *et al*., 2013), with parallel increases in body mass and WAT (Karatsoreos *et al*., 2010).

The results of the present experiments show that exposure to CUS induces a significant loss of BW (also see Iio *et al*., 2014 and Razzoli and Bartolomucci, 2016) and impairs glucose tolerance and insulin sensitivity; the data shown by Iio *et al* (2014) indicate that the chronic social defeat stress-induced reduction in BW results from decreased food intake despite a reduction in the secretion of the satiety hormone leptin by adipocytes. Leptin acts through hypothalamic receptors to inhibit food intake (Maniam *et al*., 2012) and its levels are increased after both acute stress (Konishi *et al*., 2006; Maniam and Morris, 2012; Tomiyama *et al*., 2012) and CORT treatment (Karatsoreos *et al*., 2010). While GCs promote leptin secretion from adipose tissue, they also decrease brain sensitivity to leptin (Sominsky and Spencer, 2014); at the same time, stress and GC are known to impair tissue sensitivity to insulin, a hormone that normally also signals the availability of adequate energy levels (Sominsky and Spencer, 2014). In this work, an acute stressor, imposed at the end of the CUS/CMS paradigm, reduced plasma levels of leptin in both, CUS-treated (STR) and STR+PIO-treated mice, as compared to control

(CON) and CON+PIO-treated mice, whereas insulin levels were reduced in both STR and PIO-treated mice. The decreased leptin and insulin levels in stressed animals might reflect a resistance to those peptides resulting in a hyperphagic effect. Here, it should be noted that the milder stress (CMS) given after termination of CUS (in order to facilitate behavioural testing) caused a slight recovery of BW; nevertheless, it is unlikely that the behavioural outcomes (discussed later) were markedly influenced by these changes because the mice showed impaired glucose metabolism (in the glucose tolerance test, GTT) as well as impaired insulin sensitivity (in the insulin tolerance test, ITT).

Interestingly, pioglitazone-treated mice (PIO, STR+PIO) displayed a similar response pattern to that observed in STR animals in terms of CORT levels after an acute stressor (at end of CUS/CMS exposure). Moreover, the TZD prevented the glucose intolerance and impaired insulin sensitivity induced in the STR -treated animals, despite their significantly higher BW and WAT masses. This indicates that further studies are needed to distinguish between the effects of TZDs on energy expenditure/BW and WAT mass and their effects on improving insulin sensitivity (also following STR). Understanding the underlying mechanisms will likely require consideration of the fact that chronic stress increases thermogenesis [brown adipose tissue (BAT) recruitment and increased expression and activity of the thermogenic uncoupling protein 1 (UCP1) (Kuroshima *et al*., 1984; Gao *et al*., 2003; Razzoli *et al*., 2016; Razzoli and Bartolomucci, 2016)] by activating neurons in the dorsomedial hypothalamic nucleus (DMH) which, via projections to the rostral medullary raphe region, activates sympathetic premotor neurons and triggers BAT recruitment and hyperthermia/thermogenesis in a β3-adrenoreceptor-dependent manner (Lkhagvasuren *et al*., 2011; Kataoka *et al*., 2014). At the same time, evidence showing that GCs decrease thermogenesis and the expression and activity of uncoupling protein 1 (UCP1) (Strack *et al*., 1995; Soumano *et al*., 2000; Lee *et al*., 2014), but also UCP2 (Seckl and Walker, 2004), resulting in a greater conversion of BAT into WAT, will need to be kept in mind. Although the mechanisms are obviously complex, validity for encouraging their study is provided by the fact that mice housed at thermoneutral conditions (30°C) or mice that lack β-adrenoreceptors, are resistant to diet-induced obesity (Razzoli and Bartolomucci, 2016), whereas those maintained standard housing temperatures (22°C) are more vulnerable to develop obesity (Razzoli and Bartolomucci, 2016; Sanghez *et al*., 2016) and display impaired insulin signaling. In the context of this thesis, it is interesting to note that PPARγ agonists can stimulate the

transformation of WAT to BAT (Ohno *et al*., 2012; Qiang *et al*., 2012) and increase the expression of uncoupling protein 1 (UCP1) and uncoupling protein 2 (UCP2) (Kelly *et al*., 1998; Sell *et al*., 2004).

In summary, much still needs to be learnt about the regulation of energy balance by stress. Recently, Razzoli and Bartolomucci, (2016) suggested greater focus on monitoring 1) food intake, and 2) BAT activation/thermogenesis effects. In addition, it would be important to more closely examine how stress modulates the fat browning actions that are mediated by PPARγ.

Modulation of stress-induced changes in cognitive behaviour and AD-like pathology by pioglitazone

As reviewed by Webster *et al* (2014) certain cognitive domains that are disrupted in AD (e.g. reference memory, working memory and executive function) can be studied with relative ease in mouse models of the human disease. These authors noted that, whereas some preclinical investigators have begun to examine deficits in attention, only very few have devised and used tests of episodic memory, even though this type of memory is usually first affected in AD given the pattern on neuropathological development (spreading from the hippocampus and entorhinal cortex into the medial temporal lobe, prefrontal cortex and eventually other parts of the brain). The initial impact of AD pathology in the lateral and medial temporal areas suggests that, besides episodic memory, semantic memory would also be affected during early syndromal stages of AD (Bondi *et al* 2008). In this respect, reference memory (not used in patients, but usually tested in rodents using variations of the Morris water maze), is thought to reflect semantic memory in humans (Webster *et al*., 2014). In this work, we chose to evaluate the novel object recognition memory to assess stress-induced impairments in cognition and their prevention by pioglitazone. The novel object recognition test (NOR) has gained increasing use in preclinical AD research (Grayson *et al.,* 2015), and our choice was based on the fact that its execution does not depend on external motivation, reward, or punishment and that it requires only short training/habituation times, allowing its completion in a relatively short period of time (Antunes and Biala, 2012). Moreover, NOR is known to be negatively impacted upon by chronic stress (Baker and Kim, 2002; Ivy *et al*., 2010; Eagle *et al*., 2013; Tsukahara *et al* 2015).

For the present studies, we applied a two-step objection recognition test; the first monitored object location recognition task (OLR), a hippocampus-dependent task known to be affected after CUS (Lopes *et al*., 2016), while the second measured *bona fide* novel object recognition (NOR), a task that depends on the perirhinal and entorhinal frontal cortex) (Barker and Warburton, 2011; Warburton and Brown, 2015). Importantly, analysis of the data confirmed that stress (CUS, followed by CMS during behavioural testing) impairs object recognition memory in mice, despite a high degree of inter-individual variability. Interestingly, pioglitazone produced divergent effects on recognition memory in non-stressed (control) animals, causing behavioural improvements and deficits in different subsets of mice. As mentioned earlier, the test is not known to be influenced by *external* motivation (Antunes and Biala, 2012); however, given the fact that pioglitazone caused gains in BW as well as WAT mass, likely reflected in the animals' motivation to explore in general, altered *internal* motivational state was considered as a possible confound, especially in view of previous reports that high fat diets impairs novel location recognition in mice (Heyward *et al*., 2012; Valladolid-Acebes *et al*., 2013). Accordingly, an analysis involving correlations between metabolic parameters and performance in the NOR and OLR tests was performed, but this failed to cast light on this problem, possibly due to the rather low sample sizes of the sub-groups.

Chronic exposure to STR leads to the manifestation of the two neuropathological hallmarks of AD: 1) misprocessing of amyloid precursor protein (APP), amyloid deposition, and 2) tau hyperphosphorylation, oligomerization and neurofibrillary tangle (NFT) formation. In addition, chronic STR disrupts hippocampus- and prefrontal cortex-dependent memory (Green *et al*., 2006; Jeong *et al*., 2006; Sotiropoulos *et al*., 2008a; Catania *et al*., 2009; Sotiropoulos *et al*., 2011) as well as recognition memory (Baker and Kim, 2002; Ivy *et al*., 2010; Eagle *et al*., 2013; Tsukahara *et al* 2015; Lopes *et al*., 2016) in mice and rats. In this work, biochemical analysis of the effects of STR $\pm$ PIO focused on tau protein because clinicopathological studies have established that the amount and distribution of NFTs, rather than of APP-derived peptides (which give rise to plaques), correlate with the severity and the duration of dementia (Serrano-Pozo *et al.,* 2011; Nelson *et al*., 2012).

Tau protein is necessary for stabilizing microtubules (Lee *et al*., 2001) but when it is abnormally hyperphosphorylated, the protein oligomerizes and forms neurofibrillary tangles (Iqbal *et al*., 2010; Spillantini and Goedert, 2013; Wang and Mandelkow, 2016;

Sotiropoulos and Sousa, 2016). While tau is abundant in axons, its localization in dendrites and role in synaptic plasticity (Hoover *et al*., 2010; Ittner *et al*., 2010; Kimura *et al*., 2010; Sotiropoulos *et al*., 2011; Kimura *et al*., 2013) has recently been described. Specific epitopes of tau have been reported to the pathology of AD, such as pSer202, pThr231 and pS396 (Augustinack *et al*., 2002; Lauckner *et al*., 2003; Hampel *et al*., 2005); interestingly, these same abnormally hyperphosphorylated epitopes are found to be upregulated in the hippocampus and prefrontal cortex of rodents after exposure to STR or exogenous GC treatment (Sotiropoulos *et al*., 2011; Pinheiro *et al*., 2015; Lopes *et al*., 2016). The results obtained in the present work generally confirmed the latter, but additionally, closer definition of the spatial specificity of the effects of STR was also obtained; specifically, it was observed that STR increases total tau expression (potentially increasing substrate for pathological phosphorylation) in the prefrontal cortex and ventral hippocampus and regulates AD-relevant tau phospho-epitopes (Ser202 or Thr231) in opposite directions: decreased in the prefrontal cortex and increased in the dorsal hippocampus. Interestingly, it was also found that whereas pioglitazone reduces tau-pThr231 levels in the prefrontal cortex and dorsal hippocampus of CON animals, the TZD does not prevent the occurrence of this potentially-toxic event in mice that have undergone chronic STR.

Given that pioglitazone penetrates the blood-brain-barrier rather poorly (~18% of peripherally-administered drug enters the brain; Heneka *et al*., 2005), the possibility that the dosage of pioglitazone (administered in chow) was insufficient to produce detectable changes in cognitive behaviour cannot be ruled out[14]. On the other hand, since the TZD did improve cognition, with concomitant reductions in the expression of abnormally hyperphosphorylated tau epitopes in the hippocampus and fronto-cortex, in control mice, the likelihood that the dosage applied could not override the damaging effects of the STR paradigm used should also be considered. Notwithstanding differences in test settings and parameters assessed, the present findings tend not to add support to previous reports that

[14] Since animals were group-housed in the present experiments individual intake of pioglitazone-containing food could not be determined, but individual differences in food consumption could potentially contribute to the high variability of the biochemical and behavioural data collected. Another potential caveat is that administration of drugs via chow assumes, *a priori*, that the drug is homogenously distributed and maintains its biological activity during transport and storage. The use of systemic application (intraperitoneal, subcutaneous or oral gavage) was considered during experimental design but the idea was abandoned due to the poor solubility of TZD as well as the confounds of undesired stressors (including pain) other than those that were to be imposed by design. Lastly, choice of dosage route was influenced by the reported efficacy of this route of administration in AD-related studies (Heneka *et al*., 2005; Searcy *et al*., 2012), albeit sometimes involving higher-fold doses of TZD agonists.

TZDs can prevent/reverse AD pathology at the morphobiochemical (Heneka *et al*., 2005; Pedersen *et al*., 2006; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Yu *et al*., 2015) and cognitive (Pedersen *et al*., 2006; Escribano *et al*., 2009; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Rodriguez-Rivera *et al*., 2011; Denner *et al*., 2012; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Jahrling *et al*., 2014; Chen *et al*., 2015; Yu *et al*., 2015) levels (notably all of these cited studies were conducted in transgenic mouse models of AD whereas the current experiments were carried out with wildtype mice in which AD-like pathology was induced by exposure to chronic STR). Further, the results obtained in this work are not consistent with those from clinical studies, which reported that TZDs retard memory decline in patients with mild-to-moderate AD (Watson *et al*., 2005; Risner *et al*., 2006). In this regard, it is also interesting to note that TZDs are reportedly effective in slowing memory decline in diabetic patients with mild signs of AD (Hanyu *et al*., 2009; Sato *et al*., 2011) and that TZDs, rather than another anti-diabetic drug (metformin), can reduce the risk of dementia (Heneka *et al*., 2015b). The present lack of pioglitazone treatment effects on cognition (in STR mice) are striking because the drug did prove effective at reversing some of the endocrine and metabolic effects of chronic STR.

The findings reported in the present thesis do not provide an unequivocal answer to the question: do the purported positive effects of TZDs on AD-like biomarkers and behaviours reflect direct actions on the brain rather than secondary effects on glucose metabolism in the periphery? In this context, it is relevant to note that epidemiological and clinical evidence suggests that patients with Type 2 diabetes (T2D) have an increased risk for developing AD (Merlo *et al*., 2010; Luchsinger, 2012; Pérez and Quintanilla, 2015; Heneka *et al*., 2015b). Although several preclinical and clinical research on rat models of diabetes (Yin *et al*., 2013; Fei *et al*., 2015; Ma *et al*., 2015) and diabetic patients (Hanyu *et al*., 2009; Sato *et al*., 2011), attribute the pro-mnemonic effects of TZDs to their ability to improve insulin sensitivity in the periphery, it is noteworthy that TZDs can also improve memory in non-diabetic states, e.g. non-diabetic patients and transgenic models of AD (Watson *et al*., 2005; Pedersen *et al*., 2006; Risner *et al*., 2006; Escribano *et al*., 2009; Escribano *et al*., 2010; Toledo and Inestrosa, 2010; Denner *et al*., 2012; Mandrekar-Colucci *et al*., 2012; Searcy *et al*., 2012; Jahrling *et al*., 2014; Chen *et al*., 2015; Yu *et al*., 2015). However, the picture is clouded by observations that at least one mouse transgenic line (Tg2576) displays age-dependent insulin resistance (Pedersen

and Flynn, 2004), hyperinsulinemia and impaired glucose tolerance (Rodriquez-Rivera *et al*., 2011), states that are restored to normal after TZD (rosiglitazone) treatment (Pedersen and Flynn, 2004; Rodriquez-Rivera *et al*., 2011); importantly, Pedersen *et al*., (2006) reported that rosiglitazone also ameliorates the decrease in insulin-degrading enzyme (IDE), which also seems to be related to the clearance of amyloid peptides (de la Monte, 2009), in the brain.

Other data suggesting the existence of central PPARγ include the observation that intracerebroventricular (ICV) injections of rosiglitazone ameliorate memory impairments in diabetic mice (Kariharan *et al*., 2015) as well as memory deficits induced by amyloid β (Aβ42) (Xu *et al*., 2014) and, that pharmacological blockade of central PPARγ abolishes the cognition-improving actions of rosiglitazone in mice (Denner *et al*., 2012). Complementing these observations are those showing that centrally-applied TZDs increase food intake in rats (Ryan *et al*., 2011) and the demonstration that antagonism of brain PPARγ counteracts the weight-gain effects of high fat diet and TZD treatment (Lu *et al*., 2011; Ryan *et al*., 2011). Lastly, data presented in this thesis confirm previous work (Diano *et al*., 2011) describing upregulation of PPARγ expression in the brain by an obesogenic diet. In summary, while evidence provided in this thesis and elsewhere suggests that TZDs modulate brain function by acting directly at centrally-located PPARγ, a conclusive statement cannot be made on this point which is likely to prove a difficult one to resolve (at least *in vivo*) given the intricate physiological and behavioural mechanisms that underlie the subjects of interest, namely, stress, metabolism and cognitive behaviour. Indeed, the issue is made further complex by bidirectional inter-relationships between the cognitive processes that regulate the response to stress as well as the intake of food with motivation/reward processes.

PPARγ and motivation to consume rewarding foods

Feeding is a behaviour that is essential for life and highly dependent on motivation. The hedonic properties of food drive eating, rather than its energy and make the food "rewarding" (Berridge, 2004; Berridge and Kringelbach, 2011). Reward consists of three processes: liking (derived from pleasure), wanting (derived from motivation), and learning (reward-dependent) (Berridge and Kringelbach, 2011, 2013, 2015); together, all of these processes are inter-dependent on cognitive functions such as memory, valuation and decision-making (Berridge *et al*., 2009; Higgs, 2016), all of which are relevant to

AD. Recently, PPARγ signaling has been implicated in the regulation of motivation and reward. Specifically, studies in rats showed that pioglitazone reduces motivation for heroin by attenuating the perceived rewarding properties of the drug (de Guglielmo *et al*., 2015); together with the ability of this TZD to reduce alcohol consumption (Stopponi *et al*., 2011, 2013), these data have led to the proposal that PPARγ may be a good target for preventing drug and substance abuse. Evidence supporting the latter includes the demonstration of PPARγ in the rat ventral tegmental area (VTA), specifically in the GABA-rich rostromedial tegmental nucleus (de Guglielmo *et al*., 2015), and that TZD treatment decreases extracellular dopamine levels in the shell of the nucleus accumbens (Acb) and inhibits opioid-induced stimulation of VTA dopamine neurons (de Guglielmo *et al*., 2015).

Consistent with some of the above-mentioned reports, it was reported in Chapters 3 and 4 that, pioglitazone decreases motivation and impairs operant learning for a food reward, possibly due to the drug-induced increases in BW and WAT, representative of higher energy depots. Indeed, our group previously showed that overweight and obese mice perform poorly in appetitive learning tasks, such as pavlovian conditioning and operant conditioning (Harb and Almeida, 2014), a finding indicating that the motivation to consume food is decreased when endogenous energy stores are large. These observations show that motivational and cognitive behaviours are dynamically regulated and are modulated according to fluctuating physiological and metabolic demands. Future studies in which the effects of acute, sub-chronic and chronic TZD administration on motivation to eat as a function of BW gain are compared are likely to provide interesting insights into the dynamic inter-relationships between these different domains.

Anticipation of hedonic (pleasurable) experience is a strong driver of motivation, including the motivation to seek foods that are palatable because of their taste or smell foods (Berridge and Kringelbach, 2011, 2013, 2015; Higgs, 2016); competition between hedonic drivers and homeostatic mechanisms that signal an adequate nutritional status is an important consideration in understanding the mechanisms that lead to overeating, obesity and metabolic imbalance (e.g. T2D) (Berridge, 2004; Lowe and Butryn, 2007; Beeler *et al*., 2012; Berridge and Kringelbach, 2013, 2015). In light of our data showing that pioglitazone stimulates BW and WAT gain, while maintaining normal glucose homeostasis, experiments in Chapter 4 were designed to examine whether the TZD influenced preference for isohedonic (Young and Madsen, 1963; Beeler *et al*., 2012)

sucrose- (sweet, energy-loaded) *vs.* saccharin- (sweet, energy-free) based solutions (a third bottle, containing water was also presented in this choice paradigm) in sated state but also following a period of fasting from normal solid diet. While both groups displayed a preference for saccharin, the interesting observation that, pioglitazone decreased preference for saccharin in food-deprived state was made. In fact, PIO also reduced the total consumption of solution when mice were food-deprived, a finding possibly related to the known water-retaining effects of TZDs (Guan *et al*., 2005; Basu *et al*., 2006; Bełtowski *et al*., 2013; Soccio *et al*., 2014). In summary, the results of this study suggest that PPARγ may indeed play a role in hedonic processing/motivation although the modulatory influence of energy state should not be neglected. Further, given the importance of general motivational state for optimal cognitive functioning, it will be important to examine the role of PPARγ in the regulation of overall motivational state, i.e. not that restricted to specific rewarding stimuli such as food.

Epilogue

The hypothesis tested and principal findings of the work carried out, in mice, in this thesis are illustrated in **Figure 5.1**. The experimental results demonstrate that the adverse effects of stress on glucose homeostasis and activity of the hypothalamic-pituitary-adrenal (HPA) axis are subject to modulation by agonism of PPARγ. While the hyperglycemia induced by stress was normalized after PPARγ activation by pioglitazone, the effects of the TZD on dysregulated HPA axis function were more disparate, depending on the specific aspect examined. Likewise, PPARγ activation resulted in somewhat incongruous changes in specific measures of cognitive behaviours that depend on intact hippocampal and fronto-cortical circuitries, pioglitazone decreased motivation to retrieve palatable and energy-rich food rewards. Studies aimed at examining whether any of these physiological and behavioural changes might be relevant to AD-like pathology, by monitoring the expression of total tau and its known pathological forms (hyperphosphorylation of specific epitopes) in brain areas affected by AD (hippocampus and prefrontal cortex), were relatively unsuccessful, most likely due to high inter-individual variability or due to dilution effects (if effects were limited to specific sub-populations of cells). While the behavioural changes (and to some extent, the neuroendocrine responses also), point to central sites of action of TZDs such as pioglitazone, localization of immunoreactive PPARγ in relevant brain regions was hampered by the (presumably) technical limitations

of available anti- PPARγ activation test reagents. The latter problems could be obviated in future pharmacological studies by central (intracerebral) application of PPARγ agonists and antagonists or through the comparison of outcomes in mice carrying whole body or brain-specific deletions of *PPARγ*.

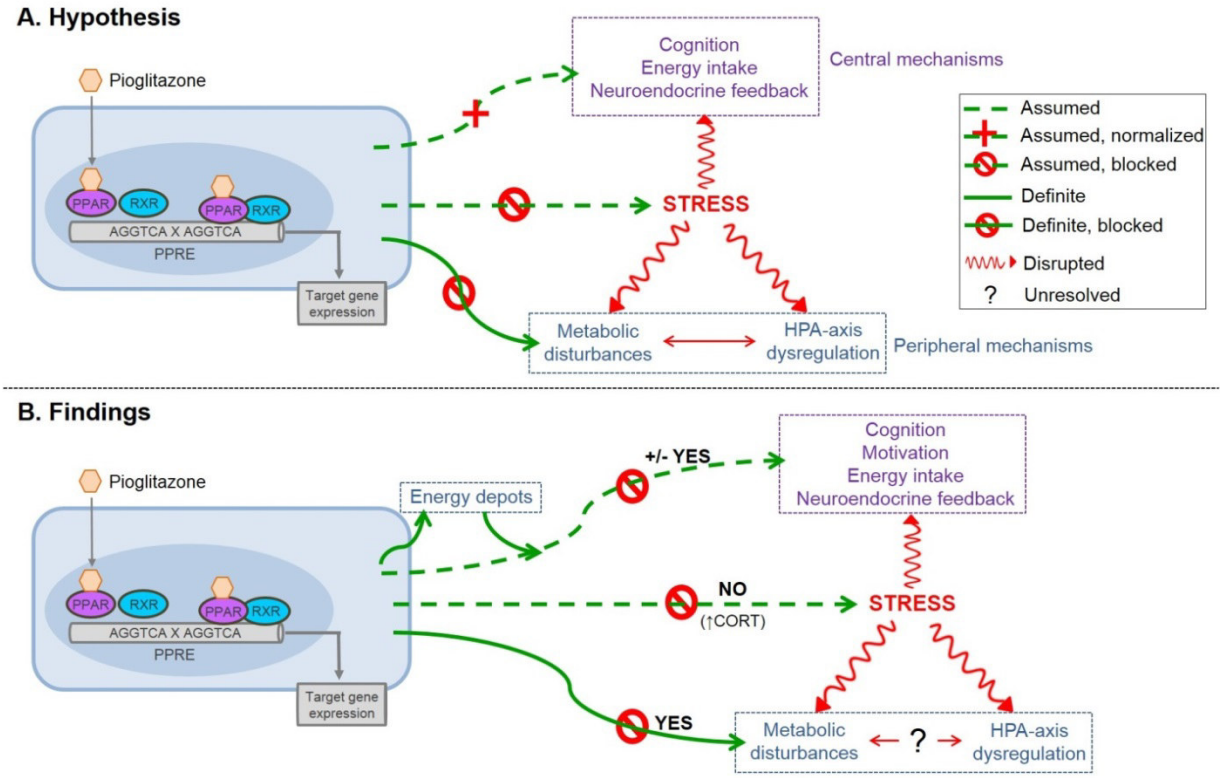


Figure 5.1. Depiction of working hypothesis and main findings of present study.
A. Hypothesis. Pioglitazone, a thiazolidinedione (TZD) that is a specific and potent agonist of the peroxisome proliferator-activated receptor γ (PPARγ), induces the transcription of genes that regulate processes within the brain, the periphery (e.g. insulin-sensitization) or at both, central and peripheral sites. Pioglitazone can potentially normalize stress-induced disruption of glucose metabolism and the activity of the hypothalamic-pituitary-adrenal (HPA) axis as well as prevent stress-induced cognitive impairment. The central effects may occur secondarily to the corrections in glucose metabolism/HPA axis function, or directly in the brain. Stress-related disorders such as insulin resistance and motivation, mood and cognitive dysfunction are interrelated at different physiological levels; recently, stress has been increasingly recognized to play a potential role in the etiopathogenesis of a serious cognitive disorder, Alzheimer's disease (AD), the incidence of which has been causally associated with metabolic dysfunction. Briefly, the hypothesis tested here was that activation of PPARγ links metabolic, endocrine and behavioural functions. To begin testing this, the work aimed to examine how pioglitazone modulates these different domains, especially under the influence of stress.
B. Findings. The efficacy of the stress paradigm used in the present study was reflected in the increased corticosterone (CORT) levels and locomotor behaviour of mice, as well as impairments in their glucose- and insulin tolerance, and reductions in body weight. Pioglitazone normalized the stress-induced anomalies in metabolism, but failed to reverse the altered levels of HPA axis activity and locomotion. At the same time, pioglitazone produced unexpected, but highly interesting bidirectional effects on cognitive behaviours that rely on the hippocampus- and cortex. Given that the drug reduced motivation for, and appetitive learning of, a food reward, while causing body weight increases, these findings raise interesting new questions about the relationship between PPARγ regulation of energy intake-related behaviours and energy storage, and how these various parameters contribute to the maintenance of metabolic homeostasis and optimal behavioural performance.

Since their discovery, PPARγ agonists have been implicated in a range of disease states, ranging from cancer and diabetes to neurodegenerative disorders. Reports that PPARγ activation improves cognitive impairment in AD patients provided the main impulse for the present work. The rationale behind these studies included the fact that agonists of PPARγ are highly effective restorers of insulin sensitivity, a condition strongly associated with cognitive impairments. Further, it was considered important to improve our understanding of the sites and mechanisms of PPARγ agonist actions (brain *vs.* periphery, or both) to add to the evidence base for their use in the treatment of neurodegenerative disorders and to contribute to the development of PPARγ-targeted drugs with greater specificity and therapeutic efficacy.

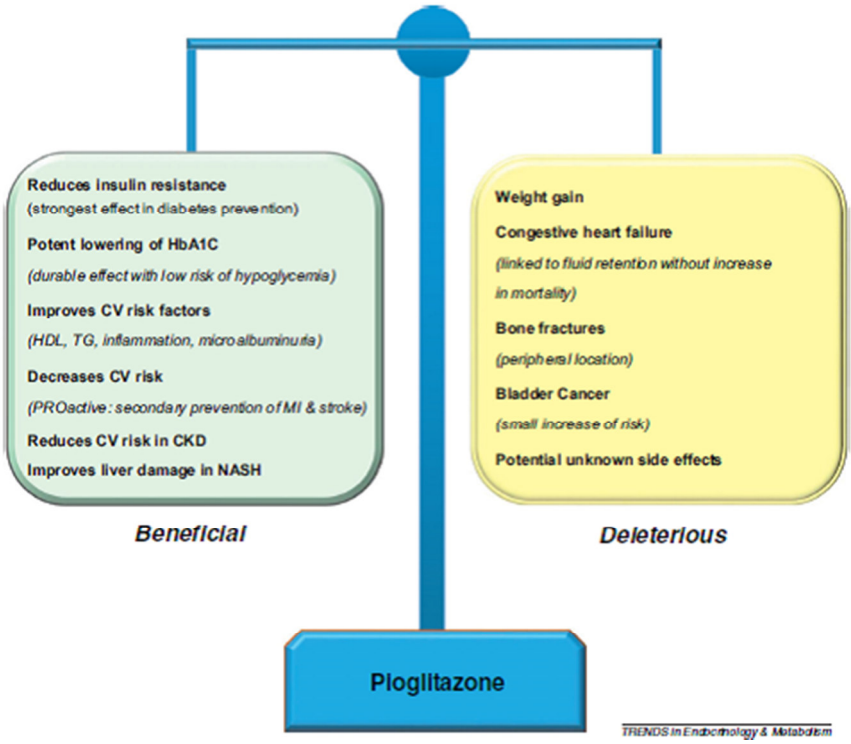


Figure 5.2. Beneficial *vs.* deleterious effects of pioglitazone treatment. Pioglitazone (PIO) improves insulin resistance but also decreases cardiovascular (CV) risk factors which in turn reduces the risk of CV mortality in type 2 diabetes (T2D) with higher risk to develop CV diseases but also in patients with chronic kidney disease (CKD). Additionally, PIO improves liver disturbances in non-alcoholic steatohepatitis (NASH). The side effects of PIO-treatment include weight gain, fluid retention that can trigger congestive heart failure, bone fractures and a small (but significant) risk to develop bladder cancer. From: **Cariou *et al.*, 2012**.

Despite their efficacious insulin-sensitizing properties, certain adverse effects of TZDs (e.g. weight gain, edema, bone loss, risk of bone fractures, congestive heart failure and bladder cancer – see Cariou *et al.*, 2012; Ahmadian *et al.*, 2013; Soccio *et al.*, 2014; Sauer

et al., 2015) have raised concerns over their use; as can be seen from **Figure 5.2**. (reproduced from Cariou *et al.,* 2012). Notably, a large meta-analysis (Colmers *et al*., 2012) reported a decreased risk for colorectal, breast and lung cancer by TZDs, including pioglitazone that has been reported to increase the risk for bladder cancer (Cariou *et al*., 2012; Soccio *et al*., 2014).

Continued use of TZDs as therapeutics clearly requires physicians to carefully weigh the potential benefits against the risks for individual patients. Meanwhile, the adverse effect evidence *vs.* the therapeutic value of TZDs for certain conditions (including brain disorders), makes a strong case for an improved understanding of the tissue-specific mechanisms of TZDs. Such work would also gain from complementary research in the area of pharmaceutical chemistry and delivery aimed at the development of novel PPARγ ligands that are tissue-specific and potent and most importantly, associated with minimal toxicity. Indeed, progress in this direction has been made with respect to compounds that specifically target PPARγ in adipose tissue; indeed, the latest generation of TZDs such as pioglitazone are associated with reduced risk for cardiovascular events as compared to earlier TZDs (e.g. rosiglitazone) (Cariou *et al*., 2012; Soccio *et al*., 2014), most likely because pioglitazone reduces blood triglycerides and unlike rosiglitazone, does not raise the levels of harmful low-density lipoproteins (LDL) and cholesterol (Chiquette *et al*., 2004); one set of authors have proposed that the lower risk of pioglitazone to trigger cardiovascular disease may be reflective of the fact that the drug pioglitazone is a weak agonist at the PPARα (Sakamoto *et al*., 2000). Unfortunately, targeting brain substrates will be more challenging than targeting peripheral tissues because of the blood-brain-barrier, which generally extrudes all but those xenobiotics that carry carefully-designed chemical modifications.

Recent advances in the knowledge of the signaling pathways that underpin obesity and insulin sensitization are beginning to inform rational design of PPARγ ligands. A promising avenue of investigation centres on phosphorylation of PPARγ at its serine 273 epitope. This phosphorylation event, usually triggered by adipocyte-produced inflammatory cytokines that activate cyclin-dependent kinase 5 (cdk5) results in repression of a subset of target genes, including that encoding adiponectin, a key insulin-sensitizing adipokine (Choi *et al*., 2010). In fact, Choi *et al*. (2010) demonstrated that the anti-diabetic effect of TZDs can be attributed to their ability to block cdk5-mediated PPARγ phosphorylation. This same group recently described SR1664, a compound that

specifically blocks cdk5-mediated phosphorylation of PPARγ with a unique transcriptional profile that allows anti-diabetic actions that are not associated with excess weight gain and fluid retention (Choi *et al*., 2011). Another study has shown that simple ablation of *cdk5* in adipose tissue has effects opposite to those desired (improved insulin sensitivity) due to compensatory increases in the activity of extracellular signal-regulated kinases (ERK) which can also phosphorylate PPARγ at serine 273 and exacerbate insulin resistance (Banks *et al*., 2014); this observation led to the suggestion that normally, cdk5 directly suppresses ERK by acting on a mitogen-activated kinase (MAPK)/ERK (MEK) site since insulin resistance in obese wild-type animals and ob/ob mice, as well as in *cdk5*$^{-/-}$ mice can be reversed by inhibition of MEK and ERK (Banks *et al*., 2014). The search for non-TZD selective PPARγ modulators (SPPARM) has also produced promising results; the compounds INT131 (now in clinical trials in T2D patients) and MBX-102 display insulin-sensitizing properties comparable to those of TZDs, but without the side effects associated with TZDs (Gregoire *et al*., 2009; Dunn *et al*., 2011; DePaoli *et al*., 2014).

In summary, even though the precise sites and mechanisms of PPARγ agonists remain open questions, it seems reasonable to be optimistic that PPARγ-targeted drugs for use to prevent or stabilize cognitive deterioration in AD, directly (by acting on brain substrates) or indirectly (by counteracting metabolic disorders and insulin insensitivity in particular), will be become available within the next 1-2 decades.

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Publications

Gazea M., Patchev A. V., Anderzhanova E., Leidmaa E., **Pissioti A.**, Flachskamm C., Almeida O. F. X., Kimura M. (in preparation). Peptide YY rescues sleep disturbances programmed by early-life obesity. (**for submission to *Journal of Neuroscience***)

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