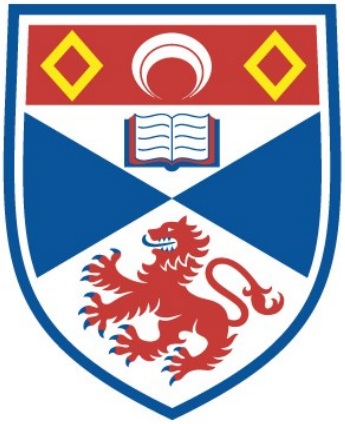
Aging, adipokines and Alzheimer's disease: exploring the neuroprotective effects of leptin and cholecystokinin

Alison Ruth Holiday

A thesis submitted for the degree of PhD
at the
University of St Andrews



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Acknowledgements

Whilst an individual writes a thesis, its completion is not possible without a wealth of support both technical and emotional. To Dr Gayle Doherty I owe the greatest thanks for her guidance, supervision, support and patience on this journey from frogs to cats. You have pushed me to be a better scientist and to do things I never would have on my own.

For their technical advice and support I would like to thank our collaborators. Thank you to Dr Jamie Ainge for his guidance on mouse behavioural tests and assistance with injections, Dr Jenni Harvey for the donation of rat brain samples, and Professor Danielle Gunn-Moore and her PhD student Lorena Sordo for donation of cat brain sections and assistance with staining and image gathering. Further to all staff past and present of the St Mary's Animal Unit who I worked alongside as both team member and student. Specific thanks to Trevor Hawkey the rodent unit NACWO who had continued patience with my multitude of questions and Isobel Maynard whom from my undergrad dissertation through to her final retirement was a joy to work and problem solve with. Finally, for technical training and advice, for being my science buddy and fuelling a coffee addiction I would like to thank my fellow PhD student, Dr Lisa Strother.

I would like to thank my colleagues in the School of Psychology and Neuroscience for their continued support from teaching me as an undergraduate through to my time as a Postgraduate. Special mentions should be made to my fellow Doherty lab students, Mizuki Morisaki and Ying Cheng, who have contributed moral and technical support as well as allowing me to moan at them when things went wrong. Thank you to Amit Chouhan, my cell culture comrade, who made every day in 'paradise' more interesting. Thank you to all the past undergraduates who allowed me to pass on my wisdom. You were fun to teach and work alongside and challenged me with new and interesting problems (of note Anna who's yellow LDH mix was green). A huge thank you also goes to Dr Brianna Vandrey, from our beginnings in the Postgraduate Society to your sacrifice (wink) helping me with the mice, you have been a constant source of positive scientific energy.

To my parents, Bob and Heather Holiday, your unfailing belief in me and support of my goals is the reason I am where I am today. Without you none of this would have been possible. Thank you for everything you do I am extremely lucky to be your daughter.

Lastly (but certainly not least) to Dr Mattias Eken, my partner in life and academic crime. You have kept me sane (and fed!) throughout these months of writing and have been a source of determination for almost four years. Jag älskar dig.

Funding

Work on leptin and cholecystokinin receptor expression in cats was supported by the ARUK Scotland Network Centre [grant number 3003_ag].

Research data access statement

Research data underpinning this thesis are available at [https://doi.org/10.17630/93223636-78f4-4452-a4f5-d248dd25f9e2]

Abstract

No current theory of ageing presents a full explanation of the ageing processes. This thesis posits focus should be on the ageing endocrine system both in healthy and pathological ageing, such as Alzheimer's disease (AD), and its treatment. Two hormones were investigated in the context of AD as potential therapeutics. Leptin has demonstrated neuroprotective and disease modifying effects both *in vitro* and *in vivo*, however it has wide ranging actions that lead to off-target effects. This thesis aimed to show the bioactive leptin peptide leptin$_{116\text{-}130}$, which may avoid these side-effects, can demonstrate the beneficial effects of leptin. Leptin$_{116\text{-}130}$ was found to mirror the neuroprotective and cognitive enhancing effects of leptin, and hence has potential for an AD therapeutic. Cholecystokinin (CCK) receptor 1 (CCK1R) agonism by A71623 was also investigated as CCK1R deficiency causes memory impairments in rats and in humans higher CCK levels correlated with decreased risk of AD. A71623 had neuroprotective effects, decreased tau phosphorylation and modulated nitric oxide. Demonstrating the potential to have disease modifying effects. Finally, as leptin and CCK have previously been shown to have synergistic action, and other co-treatments in diseases produce effects with lower concentrations, reduced side-effects and wider scope of action this therapeutic strategy was explored. A71623 co-treatment with leptin$_{116\text{-}130}$ but not leptin showed promising neuroprotective effects. Changes in these drugs receptors, ObR and CCK1R, studied using the ageing SHSY-5Y model, rat cortex and healthy and cognitive decline syndrome cats, show co-treatment may generate better long-term efficacy due to differing changes in receptor expression by brain region both with age and onset of an AD-like disease. These results demonstrate for the first time the promise of leptin$_{116\text{-}130}$ and CCK1R agonism in the treatment of AD and as such greater understanding of the endocrine system with age may lead to better treatments for age-related diseases.

CONTENTS

1 TABLE OF FIGURES

2 ABBREVIATIONS

Abbreviation	Definition
5-fdu	5-fluorodeoxyuridine
ABAD	Amyloid binding alcohol dehydrogenase
AC	Adenyl cyclase
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AGE	Advanced glycation end product
AIF	Apoptosis-inducing factor
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BACE 1	β-site amyloid precursor protein cleaving enzyme 1
BBB	Blood-brain barrier
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CB_5R	Cytochrome b5 reductase
CCK	Cholecystokinin
CCK1R	Cholecystokinin receptor 1
CCK2R	Cholecystokinin receptor 2
CDS	Feline cognitive decline syndrome
c-fos	Fos proto-oncogene
CNS	Central nervous system
CoQ	Coenzyme Q
CRD	Cytokine receptor homologous domain
CSF	Cerebrospinal fluid
CTCF	Corrected total cell fluorescence
Cu	Copper chloride
CV	Crystal violet
Cyt C	Cytochrome c
DAF-2DA	4,5-Diaminofluorescein
DAG	Diacylyglycerol
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDL	Extensor digitorum longus muscle
EIF4E-BP	Eukaryotic translation initiation factor 4E binding protein
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ER	Estrogen receptor

ERK	Extracellular signal-related kinase
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FAK	Focal adhesion kinase
FCS	Fortified calf serum
Fn3	Fibronectin type 3 domain
FungiBact	Penicillin-streptomycin-neomycin
GDPH	Glycerol-3-phosphase dehydrogenase
GPA	Glutathione peroxidase A
GPCR	G-protein coupled receptor
Grb	Growth factor receptor-bound protein
GSNOR	S-nitrosoglutathione
H_2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
HBSS	Hank's buffered salt solution
HSB	Horse serum block
HSF	Heatshock transcription factor
HSP	Heatshock protein
ICC	Immunocytochemistry
ICV	Intracerebroventricular
Ig	Immunoglobulin-like domain
IGF1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IP	Intraperitoneal
IRS	Insulin receptor substrate
IκB	Initiator of κB
Jak	Janus kinase
JNK	c-jun NH_2-terminal kinase
LDH	Lactate dehydrogenase
L-NIO	N-(1-lminoethyl)-L-ornithnie dihydrochloride
LRP-2	Lipoprotein receptor-related protein-2
LTD	Long-term depression
LTL	Leukocyte telomere length
LTP	Long-term potentiation
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MCI	Mild Cognitive Impairment
M-CSF	Macrophage colony-stimulating factor
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NBF	Neutral buffered formalin
NBT	Nitroblue tetrazolium
NFT	Tau neurofibrillary tangles
NF-κB	Nuclear factor-κB

NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOR	Novel object recognition task
NOS	Nitric oxide synthase
NOS1/ nNOS	Neuronal NOS
NOS2/ iNOS	Inducuble NOS
NOS3/ eNOS	Endothelial NOS
NS	Nitrosative stress
ObR	Leptin receptor
OC	Object context recognition task
OLETF	Otsuka Long-Evans Tokushima Fatty (rat)
OP	Object place recognition task
OPC	Object place context recognition task
OPT	Object place temporal order task
OS	Oxidative stress
OVX	Ovariectomised
P450	Cytochrome P450
P70S6K	Ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PenStrep	Penicillin streptomycin
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
Prx3	Peroxiredoxin 3
PSN1	Presenilin 1
p-tau	Phosphorylated tau
PTEN	Phosphate and tensin homolog
PYK	Protein tyrosine kinase
Raf	Rapidly accelerated fibrosarcoma
RAGE	Receptor for AGE
RNS	Reactive Nitrogen species
ROS	Reactive Oxygen Species
SAβ-gal	Senescence associated β-galactosidase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Ser262	Serine 262
SGD	Combined serum glucose deprivation
SIRT	Sirtuins
SMTC	S-methyl-L-thiocitrulline
SMUT	S-methylisothiourea
SNIP	SNAP-25-interacting protein
SOCS	Suppressor of cytokine signalling
SOD	Superoxide dismutase
SR2	Serum replacement 2
STAT	Signal transducer and activator of transcription proteins
TBS	Tris-buffered saline
TFAM	Mitochondrial transcription factor A

3 INTRODUCTION

3.1 WHAT IS AGEING?

Ageing is a deleterious biological process which progressively increases the likelihood of death (Comfort, 1956). There is a decline in bodily functions and an accumulation of damage to organs, tissues and cells over an organism's lifespan caused by both internal and external factors. Four criteria, which any age-related change should meet before it is defined as part of the ageing process, are: universality, intrinsicality, progressiveness and deleteriousness (Strehler, 1962). The underlying assumption is there are gradual changes in an organism's structure with time, not explained by disease or accident, which lead to the increased probability of death. Universality means these changes must occur in all members of a species, eliminating specific hereditary and environmental diseases. Intrinsicality rules out external factors which may contribute to ageing, so changes must come from within the organism. This removes modifiable environmental changes from the ageing equation. The third criteria, progressiveness, encompasses the idea that ageing is a process rather than sudden event, therefore onset should be gradual and cumulative. The final criteria, deleteriousness, is the most characteristic change occurring in ageing. With every ageing individual a decline in function is observed leading to an increased mortality rate. The first three criteria (universality, intrinsicality and progressiveness) must contribute to this increased probability of death. This helps rule out purely developmental processes as they contribute to increased adaptability and survival capacity (Strehler, 1962). Given this, the definition of ageing can be updated. Biological ageing is more specifically defined as progressive decline in function, occurring post-maturation at the individual, system and cellular level (Bousquet *et al.*, 2015). Note that here biological ageing is referred to specifically as theories indicate humans also undergo psychological and social ageing. These refer to a human's ability to adapt to the ageing process (psychological) and how ageing changes their role in society (social). Within biological ageing we might also subdivide into healthy and pathological ageing. Whilst healthy ageing conforms to the definitions given, pathological ageing refers to a rapidly progressing impairment of functions in the body which lead to premature death (Dziechciaz and Filip, 2014). Examples of these age-related diseases are atherosclerosis, obesity,

cancer, osteoporosis and neurodegenerative diseases such as Parkinson's and Alzheimer's disease (AD; see 3.3). These are considered to be diseases which have ageing as a risk factor, as opposed to being a part of natural ageing because they violate the rules of healthy ageing whereby sufferers no longer have independence in daily living activities (Rattan, 2013), they accelerate the ageing process (e.g. neurodegeneration) (Toepper, 2017), and increase the risk of sudden (Abildstrom *et al.*, 2002; Ayesta *et al.*, 2018) or premature death (Ryngach and Vlasyk, 2018).

Whilst an accurate definition of ageing is useful for helping us identify processes or changes as part of ageing it tells us little about the ageing phenotype. In humans changes occur across many systems including: circulatory, respiratory, digestive, urogenital, musculoskeletal-locomotor, nervous, sense, and skin and appendages (Dziechciaz and Filip, 2014). These system changes lead to more widely known characteristics of ageing such as increased risk of developing non-communicable disease (e.g. cardiovascular, macular degeneration and dementias) (Global Burden of Metabolic Risk Factors for Chronic Diseases Collaboration, 2014; Simon, Hollander and McMichael, 2015), reduced locomotive abilities (Ihlen, 2014; McCrum *et al.*, 2018) and changes in skin appearance (e.g. wrinkles and age spots) (Rinnerthaler *et al.*, 2013). Across species there are similarities in the system changes seen with age and even the ageing phenotype. As such in the exploration of ageing and age-related diseases animal models are employed in order to better understand these processes. Common animal models include rodents (rats and mice), fish, birds and amphibians. These models allow us to study *in vivo* changes within an ageing system; the impact of environmental and genetic manipulations; and efficacy of pharmacological interventions (Mitchell *et al.*, 2015). However, there are also many differences in the ageing process across species which may explain why some non-communicable disease do not naturally occur in some species (**Table 1**).

	Similarities to humans	Differences to humans
Rats/mice	Steady decline in health with age. Deterioration in appearance, posture, muscle tone and mobility (Phillips *et al.*, 2010) Female rats demonstrate gradual decrease in serum estradiol (Moorthy *et al.*, 2005)	Mice have longer telomeres and telomerase activity (Gomes *et al.*, 2011) Laboratory models are usually inbred for genetic homogeneity, as such results from this work may not be generalisable (Miller *et al.*, 1999)
Fish	Build-up of senescence-associated beta-galactosidase activity and oxidised protein accumulation in muscle (Kishi *et al.*, 2003) Approximately 70% of human genes have an orthologue in the Zebrafish genome (Barbazuk *et al.*, 2000; Howe *et al.*, 2013)	Zebrafish have similar length telomeres to humans but use telomerase (Anchelin *et al.*, 2013) No build-up of lipofuscin granules (age pigments) (Kishi *et al.*, 2003)
Birds	For some species probability of death increases with time and reproductive success declines with age (Travin and Feniouk, 2016)	Some demonstrate increased reproductive success with age and accumulate advanced glycation end-products more slowly (Holmes, Flückiger and Austad, 2001)
Amphibians	Build-up of age-related pigments (Tanaka *et al.*, 1974; Kara, 1994)	Xenopus laevis demonstrate no changes in in fertility and skin substance with age and no involution of organs (Brocas and Verzar, 1961)

Table 1. Similarities and differences between human ageing and common laboratory models used to study ageing.

Given this, and the fact that many of these animals are used in the study of ageing, the specific underlying mechanisms of healthy ageing in humans still elude our understanding.

3.2 How do we age?

There are many theories attempting to explain the mechanisms and causes of ageing. Among these the common theme is damage caused on a cellular level by external sources or a build-up of by-products from normal bodily processes leading to eventual malfunctioning. The most basic of these theories posits that a toxic environment for cells is generated from waste product accumulation after

bacterial and viral infections causing cellular malfunction and death (Park and Yeo, 2013). This is characterised in the typical ageing phenotypes seen in rats and humans whereby with age there is a steady decline in heath with deterioration in appearance, posture, muscle tone and mobility (Phillips *et al.*, 2010). This is termed the wear and tear theory of ageing.

3.2.1 Wear and tear theory: build-up of toxic by-products from extrinsic sources causes cell death.

One of the by-products highlighted in this theory are age pigments. Age pigments, for example, lipofuscin granules, form due to the incomplete degradation of debris from lysosymes. These accumulate with age and have been implicated in age-related diseases, such as macular degeneration. An increase in these pigments beyond the normal level is seen in such diseases (Feldman *et al.*, 2015). Their presence in healthy ageing is explained by long-term autophagy of old organelles (Iwasaki and Inomata, 1988). In retinal pigment epithelial cells, light exposure in lipofuscin granule-fed cells causes a decrease in cell viability (Davies *et al.*, 2001). Hence, over time a build-up of granules may contribute to cell death and therefore decreased visual ability with age.

This theory automatically opposes the criteria Strehler set out for normal ageing as it assumes ageing has an external rather than intrinsic cause. Therefore, by this theory ageing should be reduced by living in a manner wherein the organism is protected from external harm to cells. Whilst the human population has increased its lifespan with improved healthcare, ageing has not been negated and access to better healthcare does not necessarily change the ageing process. Instead it extends life or health-span such that adults can live healthier for longer but continue on the ageing trajectory. Furthermore, in animals in captivity, where there is less risk of disease and better healthcare compared to their counterparts in the wild, not all species demonstrate an increase in lifespan. For example, whilst red deer see an extension in lifespan when held in captivity versus being in the wild, roe deer see the opposite trend (Müller *et al.*, 2009). Further, long-lived amphibian species such as the *Xenopus laevis* highlight a gap in this theory as they demonstrate no changes in fertility and skin substance with age and no involution of organs seen in other species (Brocas and Verzar, 1961). Despite their negligible

senescence some age-related changes have been identified such as a build-up of melanin in regions of high metabolism (Tanaka *et al.*, 1974; Kara, 1994). Although how this effects the frogs or leads to eventual death is unclear.

3.2.2 Rate of living theory: Higher metabolic rate reduces lifespan

Whilst wear and tear focusses solely on damage due to the external environment other theories look at internal causes which may result in maladaptive processing or damage from normal processes. One of the most well-known theories of ageing is the rate of living theory (Rubner, 1908) which posits large animals, such as elephants, live longer than small animals such as mice (Blagosklonny, 2013). As small animals have greater basal rate of oxygen metabolism than large animals it was hypothesized that this leads to a shorter lifespan (Muller *et al.*, 2007). However, this theory does not hold true across different species, for example, elephants with a basal metabolic rate of 0.6W (Heusner, 1991), have a shorter life span than the smaller Sulphur crested cockatoo with a higher metabolic rate of 3.14W (Thomas, 2007). Additionally, animals of a similar size within a species can have differing metabolic rates so do not necessarily adhere to this theory. For example, birds fall into two categories of lifespan short-lived with varying metabolic rates and longer-lived with high metabolic rate, body temperature and blood glucose levels. According to both the oxidative damage theory (see 3.2.3) and the glycation theory of ageing this second group of birds should be short-lived and have rapid senescence (see 3.2.5). However, they undergo very gradual senescence and some demonstrate increased reproductive success with age (Holmes, Flückiger and Austad, 2001). Although this theory did not demonstrate a full picture of ageing it highlighted the importance of oxygen metabolism.

3.2.3 Oxidative damage theory: Accumulation of reactive oxygen and nitrogen species overcome antioxidant capacity leading to oxidative stress, cell damage and death.

It is now known that the accumulation of by-products of energy metabolism can have deleterious effects on cells and it is proposed that this is central to the emergence of an ageing phenotype (Harman, 1956) . These free radicals are generated in proportion to metabolic rate (Brys,

Vanfleteren and Braeckman, 2007), so that a higher metabolic rate leads to a higher free radical production. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) were also highlighted as contributing to damage associated with ageing. The electron transport chain (ETC) in the mitochondria is a major source of ROS generation within cells. It uses single electron transfer in the process of energy metabolism and uses ROS and RNS such as hydrogen peroxide and nitric oxide (NO) during this process. Whilst these products are often reduced to water or reused in the process, it is an imperfect system. One quality often attributed to the ETC is 'leakiness' of the chain which allows these damaging products to escape and cause damage throughout the cell (Figure 1).

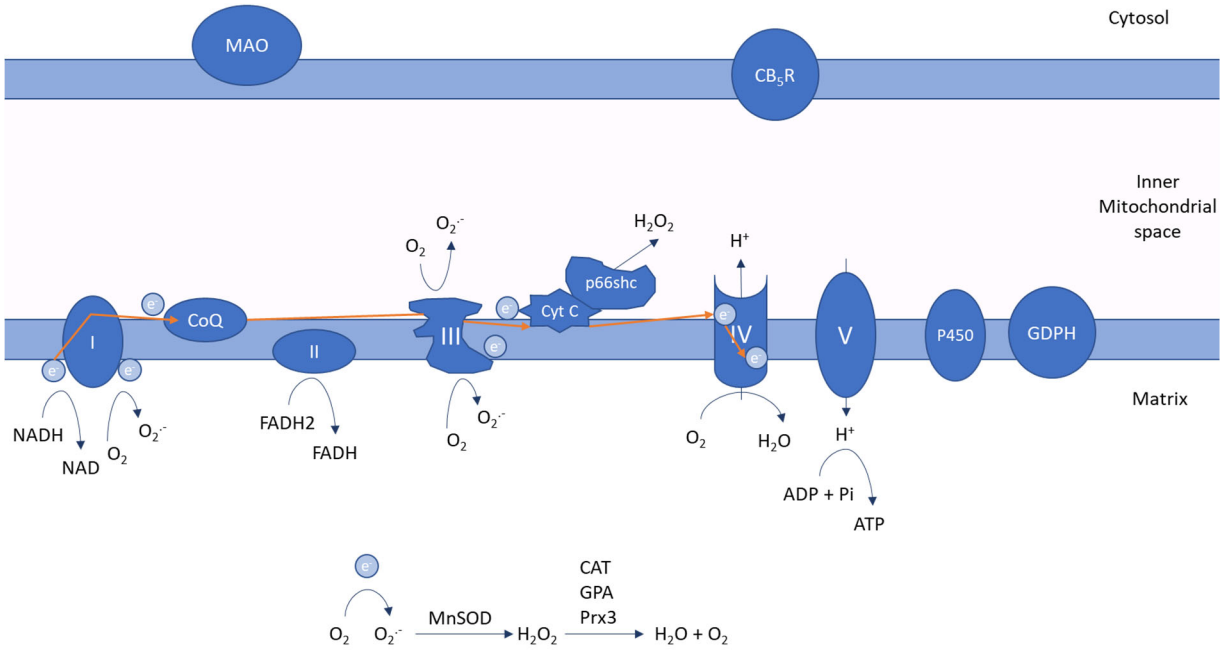

Figure 1. Reactive oxygen species generation in the mitochondrial electron transport chain. ROS are produced at several points in the ETC in the process of making ATP. These are usually converted to water and oxygen via superoxide dismutase (SOD) however this is an imperfect system. MAO = Monoamine oxidase, CB_5R = Cytochrome b5 reductase, I = Complex I, II = Complex II, III = Complex III, IV = Complex IV, V = Complex V, CoQ = Coenzyme Q, Cyt C = Cytochrome c, p66shc = protein of 66kDa with Src homologous-collagen homologue, P450 = Cytochrome P450, GDPH = Glycerol-3-phosphase dehydrogenase, NAD = Nicotinamide adenine dinucleotide (oxidised), NADH = Nicotinamide adenine dinucleotide (reduced), FADH = Flavin adenine dinucleotide, ADP = Adenosine diphosphate, ATP = Adenosine triphosphate, MnSOD = Mitochondrial superoxide dismutase, CAT = Catalase, GPA = Glutathione peroxidase A, Prx3 = Peroxiredoxin 3, e^- = electron.

An imbalance between the generation of ROS and the ability of antioxidant defences to deal with them, is termed oxidative stress (OS) which causes aggregation, oxidation and fragmentation of

proteins, lipids and DNA (Feeney and Schöneich, 2012; Ivanova and Yankova, 2013). OS is particularly important in neurons as these cells do not mitose, lack the extensive repair mechanisms of other cell types (Iyama, Wilson and III, 2013) and have a high metabolic rate (Sickmann and Waagepetersen, 2014). Thus, OS is hypothesised to contribute to the increased risk of neurodegeneration with age. Inducing OS causes cognitive impairment in young (3 months) and old rats but this is exacerbated in the old group (24-25 months) (Fukui *et al.*, 2002, 2006) so there is some mechanism of oxidative stress sensitivity with age. This is supported by work that found oxidative damage to nucleic acids increased with age in the hippocampus (Liu *et al.*, 2002). Thus, an area of already high OS would be more easily overloaded by an increase.

This theory also posits that an overabundance of RNS can cause system stress in the form of nitrosative stress (NS) leading to, for example, apoptosis and glial cell activation (Drechsel *et al.*, 2012). However, more recent work has called into question the role of NO in this process. In both mouse and human hippocampus levels of S-nitrosoglutathione reductase (GSNOR) increase with age. This enzyme metabolises NO and regulates s-nitrosation, and mice overexpressing GSNOR demonstrate cognitive impairment, LTP defects and decreased dendrite spine density. On the other hand, upregulation of NO signalling pathways, conversely to the free-radical theory, were able to rescue cognitive impairments in these mice (Zhang *et al.*, 2017). The role of NO in neural plasticity, learning and memory is well established (Pavesi, Heldt and Fletcher, 2013; Moosavi *et al.*, 2014; Pitsikas, 2015) as well as its role in the inflammatory response (Guzik, Korbut and Adamek-Guzik, 2003; Leppänen, Tuominen and Moilanen, 2014; Ljubisavljevic *et al.*, 2014). Over-production of NO can be neurotoxic (Calabrese *et al.*, 2007) and occurs naturally as part of the inflammatory response but is linked to pathogenesis in several neurodegenerative diseases (Togo, Katsuse and Iseki, 2004; Colombo *et al.*, 2012; Khandare, Ankulu and Aparna, 2013; Tripathy, Chakraborty and Mohanakumar, 2015). In healthy ageing excessive NO production as part of neuroinflammation leads to microglial activation which may contribute to increased age-related risk of neurodegeneration (Yuste *et al.*, 2015). Hence, NO is often referred to as

a Janus molecule due to its vital role in survival functions and implications in disease and senescence (Calabrese *et al.*, 2009).

Free radical theory can counteract the problems of high-metabolism organisms presenting long lifespans as it considers an organism's capacity to counteract free radicals. For example, in the short-lived amphibian species, Rana Nigromaculata, the antioxidant enzymes SOD, catalase and glutathione peroxidase decrease in an age-related matter with livers from 10-year-old animals showing 40-80% less activity compared to 3-year-old livers. Alongside this, ROS damage such as inorganic peroxide concentrations (e.g. superoxide) and thiobarbituric acid reactive substances (readily reacts with end products of lipid peroxidation such as malondialdehyde (Janero, 1990)), greatly increase. This suggests an inverse correlative relationship between antioxidants in the liver and ROS damage (Kashiwagi *et al.*, 2005). Further, in birds it has previously been suggested that longer-lived species have a higher antioxidant capacity compared to shorter-lived ones thus explaining why they live longer despite a higher metabolic rate. Nevertheless, a comparison of short-lived quail and long-lived parrots showed no differences in antioxidant capacity (Montgomery, Buttemer and Hulbert, 2012) nor any differences in ROS production (Montgomery, Hulbert and Buttemer, 2012). In comparison to mammals, birds organs are less susceptible to free radical damage (Pamplona *et al.*, 1999), produce less free radicals and have less leaky mitochondria (Barja *et al.*, 1994) but this does not guarantee them a longer lifespan or reduced impact of ageing in comparison.

It is also well documented that life span increases through calorie restriction and exercise, both of which increase OS (Schulz *et al.*, 2007; Afanas'ev, 2010), which argues against free-radical theory. Furthermore this theory implies increasing antioxidant levels should negate the impact of free-radicals on ageing, however, antioxidant supplementation decreases lifespan in the shirt-tailed field vole (Selman *et al.*, 2013). Similarly vitamin E supplementation reduces lifespan in humans (Miller *et al.*, 2005).

To date, the only common ageing pathway identified in Drosophila, mice and humans involves the downregulation of an ETC gene set. These genes code for a mitochondrial enzyme which produces free radicals (Zahn *et al.*, 2006; Kim, 2007). Knocking down homologous components of this gene set in C. *elegans* increases lifespan (Lee *et al.*, 2002), hence there appears to be some relation between ETC functionality, oxidative damage and ageing although the exact relationship is not yet understood. It is possible that the free radical theory focusses too narrowly on the impact of OS on the body whilst not fully considering slow but gradual modifications occurring throughout the body caused by other reactive metabolites (Golubev, Hanson and Gladyshev, 2017). These metabolites can form during increased energy demands from cells leading to a dependence on glycolysis, such as in ageing or metabolic disorders e.g. diabetes (Fleming *et al.*, 2010). An example of this is the dicarbonyl species methylglyoxal which can cause spontaneous modifications, in the form of glycation, to slowly renewing macromolecules, such as protein and DNA, leading to a build-up of damage independent of OS (Rabbani, Xue and Thornalley, 2016).

3.2.4 Telomere theory: Reaching the Hayflick limit causes DNA degradation and cell senescence.

Whilst only one common ageing pathway has been identified across widely used laboratory models and humans, other basic functions remain similar. An example of this can be seen in cell replication, more specifically in mechanisms to conserve and protect DNA during cell replication. The ends of DNA strands contain non-coding regions termed telomeres. Due to limitations in replication machinery the end of DNA is mistaken for a double-strand break and is unable to be completely copied (Gilson and Ségal-Bendirdjian, 2010). Hence telomeres protect the loss of coding regions of DNA as this section can go unreplicated without impact on the functionality of the cell. However, the effectiveness of this mechanism is limited by the length of telomeres and frequency of replication (Hayflick and Moorhead, 1961; Hayflick, 1965; Corey, 2009). Once telomeres are completely degraded new cells will have reduced function, as further replications will result in a loss of coding regions, and would be considered aged (Watson, 1972; Olovnikov, 1973). This finite ability of cells to replicate is termed the Hayflick limit (Hayflick and Moorhead, 1961). Many animals (e.g. Zebrafish, mice, birds) produce the

enzyme telomerase which can extend telomere sequences. This extends chromosomal life by increasing the number of replications needed until senescence (Greider and Blackburn, 1985). Human somatic cells do not produce telomerase and hence telomere shortening and chromosomal damage is implicated in cellular ageing (Harley, Futcher and Greider, 1990).

Absolute telomere length in humans is 10-15 kb but in mice it is 50-150 kb (Ozturk, Sozen and Demir, 2014). It is well known that humans live much longer than mice, but with shorter telomeres and no telomerase activity human cells should reach senescence much sooner. Smaller short-lived mammalian species have relatively long telomeres and high telomerase activity, compared to larger long-lived species which possess short telomeres and suppression of telomerase. This latter strategy suppresses tumour formation, whilst telomeres are still long enough to survive shortening with cell divisions (Gomes *et al.*, 2011). Short telomeres and suppression of telomerase activity leads to replicative senescence whereby cells cease growth and cannot re-enter the cell cycle, become resistant to apoptosis and acquire changed differentiated functions. This state can be triggered by for example, DNA damage which might lead to mutations and cancer (Campisi, 2000).

For post-mitotic cells, such as neurons or myocytes, strict application of telomeric theory means these would not age as their telomere length is consistent post-differentiation. However, these cells do age. One explanation is that ageing of supporting structures causes ageing of these cell types. Telomere length in leukocyte cells has shown a correlation with many tissues as they age (Daniali *et al.*, 2013). In fact average leukocyte telomere length (LTL) inversely correlates with age and is linked to cardiovascular disease (von Zglinicki *et al.*, 2000; Samani *et al.*, 2001), cerebral subcortical brain atrophy and white matter hyperintensities (Wikgren *et al.*, 2014). However, LTL has been suggested to be a marker of left ventricle ageing but no direct link could be uncovered in a direct correlation of telomere length and myocardial structural changes (Akasheva *et al.*, 2015). Decreased LTL has been associated with decreased volume of many brain regions including the hippocampus (King *et al.*, 2014) and faster LTL attrition in young adulthood is associated with poorer cognitive function in midlife (Cohen-

Manheim *et al.*, 2016) however relative LTL and telomere length in brain tissue did not show significant correlation (Dlouha *et al.*, 2014). Whilst this indicates that telomere shortening in interacting areas can impact non-mitotic cells it does not necessarily show that ageing in one dictates ageing, or telomere shortening, in the other. Hence telomere shortening alone cannot provide a unifying theory of ageing.

Interestingly links have also been made between telomere length and OS. OS (Coluzzi *et al.*, 2014; Ludlow *et al.*, 2014) and inflammation (Aulinas *et al.*, 2015; J. Zhang *et al.*, 2016; Lee *et al.*, 2016) could lead to accelerated telomere loss hence telomere length could be an indicator of chronic OS (Balasubramanyam *et al.*, 2010). In patients with premature coronary artery disease there is a trend towards shorter LTL than healthy controls and reduced antioxidant capacity (Tian *et al.*, 2017). Higher dietary total antioxidant capacity is positively correlated with longer telomeres in children (García-Calzón *et al.*, 2015), indicating a potential link between telomere length and OS capacity. This link has also been demonstrated in other species with Tree Swallows demonstrating a trade-off between reproduction and higher OS and survival. Animals with longer telomeres produced lighter nestlings which would be less likely to survive, however these adult birds were more likely to survive from year-to-year than individuals with shorter telomeres, lower plasma antioxidant capacity and higher ROS (Ouyang *et al.*, 2016). Therefore, by bringing these existing theories together, we may start to create a more convincing theory of ageing, but this still leaves the problem of reactive metabolites other than ROS and RNS which cause the slow build-up of damage long term, such as through glycation.

3.2.5 Glycation theory: Advanced Glycation End-product protein modification induces cell senescence.

Glycation is the process whereby glucose binds to proteins and lipids, modifying their structure and function. Glycation leads to the production of advanced glycation end products (AGEs), which are implicated in ageing (Yamagishi, 2012; Singh *et al.*, 2014). AGEs are a common occurrence in both ageing and diabetes. Whilst in healthy adults AGE crosslinks accumulate over time, patients with type 2 diabetes (T2D) have abnormally high glucose levels, a surplus of which can glycate proteins (Gautieri

et al., 2014). In fact the group of conditions termed diabetes mellitus are characterised by hyperglycemia, which is associated with organ and tissue damage and dysfunction (Alam, Asghar and Azmi, 2014). Due to this high-sugar state diabetes patients demonstrate an accelerated ageing phenotype which includes shortened telomeres, decreased mitochondrial DNA content, hypoadiponectinemia (reduced adiponectin blood-levels) and increased oxidative stress (Monickaraj *et al.*, 2012; Ma *et al.*, 2013), as well as earlier loss of leg muscle strength and functional capacity (Park *et al.*, 2007; Leenders *et al.*, 2013).

Interventions designed to counteract the build-up of AGEs include calorific restriction and reduced sugar intake. As such, blocking glucose metabolism and glycolysis increases maximum lifespan by 25% and mean lifespan by 17% in *Caenorhabditis elegans* (Schulz *et al.*, 2007). Conversely, exposing these animals to a hyperglycaemic environment decreased lifespan and this was seen to be caused by a downregulation of DAF-16/FOXO activity and aquaporin gene expression (Lee, Murphy and Kenyon, 2009). Similarly, in mammalian cell culture, elevated glucose induces senescence (measured by detection of senescence associated β-galactosidase (SAβ-gal) positivity); alters downstream mediators of sirtuins (SIRT) e.g. FOXO1; and upregulates histone acetylase p300. This leads to an attenuation of FOXO1 DNA binding, reducing SOD levels, hence reducing antioxidant capacity, and increases OS (Mortuza *et al.*, 2013). Hyperglycaemia also downregulates SIRT3, which is linked to enhanced human lifespan. Expression changes of single-nucleotide polymorphisms in exon2-3 position 477 and VNTR in intron 5 of SIRT3, impact survival rates with age in adults and further lower expression of SIRT demonstrated decreased longevity in humans (Rose *et al.*, 2003; Bellizzi *et al.*, 2005). In human cell cultures overexpression of SIRT3 reduces FOXO1 acetylation, increasing expression of mitochondrial SOD, and antagonises cellular senescence as measured by delayed SAβ-gal staining, senescence-associated heterochromatin foci formation, and p16(INK4A) expression which is thought to be a genetic marker of ageing as its expression is increased in most tissues with age (Zhang *et al.*, 2013). However other animal systems seem to contradict the importance of sugar in ageing. Long-lived birds have high metabolic rate, body temperature and blood glucose levels which according to the glycation theory of

ageing this group of birds should be short-lived and have rapid senescence. However, In comparison to mammals, birds accumulate AGEs more slowly (Holmes, Flückiger and Austad, 2001).This is demonstrated by increasing glucose concentrations in chicken and human serum albumin. Whilst glycation increases with glucose concentration in both species, the increase is significantly less in chickens and is unaffected by temperature, whereas humans see increased glycation with increased temperature. This suggests there are underlying structural differences allowing these birds to accumulate AGEs more slowly (Zuck *et al.*, 2017). As such this work indicates assessing changes at a system rather than cellular level may give important insights into ageing.

3.2.6 Endocrine theory: Decline in hormonal functioning with age causes system-wide deterioration and ageing.

In contrast to the above-mentioned theories which tend to focus on cellular level causes of ageing the endocrine theory of ageing adapts a more system wide approach. The endocrine theory posits that hormone secretion and sensitivity decrease with age. Due to their vital roles in; maintaining homeostasis, regulating reproduction, metabolism, nutrition and growth, this decline in effect leads to gradual system dysfunction. DAF-16/FOXO are downstream of the insulin and insulin-like growth factor 1 (IGF1) endocrine pathway and single gene mutations in this pathway have been shown to extend lifespan in worms, flies and mammals (Russell and Kahn, 2007). IGF1 usually stimulates the uptake of glucose from the bloodstream dose-dependently, indicating that a decrease in IGF1 leads to an increase in circulating glycation substrates and hence increased AGEs (Sroga, Wu and Vashishth, 2015). Avian species also possess IGFs which have both similarities (e.g. correlations between IGF1 levels and growth (Vasilatos-Younken and Scanes, 1991)) and differences (e.g. IGF1 is available in free-form in birds (McMurtry, Francis and Upton, 1997)) to mammalian versions, but also differ in effect across avian species (Vasilatos-Younken and Scanes, 1991). It may be that these differences are key in differing accumulation rates of AGEs. Studying the glucose uptake of skeletal muscle in English sparrows has shown that increasing IGF1 did not increase glucose uptake as it does in mammals, although in this study human IGF1 was used in treatments (Sweazea and Braun, 2005).

IGF1 has also been shown to stimulate ROS production and can in turn be regulated by ROS (Lee and Kim, 2018). IGF1 has been seen to induce vascular smooth muscle cell proliferation through the production of ROS, mediated by NADPH oxidase 4 and Rac1 (Meng, Lv and Fang, 2008) and vice versa, application of H_2O_2 to vascular smooth muscle cell increased synthesis of IGF1 (Delafontaine and Ku, 1997). This suggests a mechanism whereby IGF1 may use ROS in signalling under normal conditions, but an overproduction of ROS may result in IGF1 increase and dysregulation. Moreover in SHSY-5Y cell culture IGF1 protected cells from amyloid beta (Aβ) cytotoxicity by reducing ROS and activating the PI3K/Akt cell survival pathway (Z. Wang *et al.*, 2017) demonstrating that it may also be a regulator of ROS.

We have seen that OS can lead to accelerated telomere loss and longer telomeres are an indicator of antioxidant capacity. Further downregulation of DAF-16/FOXO, a downstream component of the IGF1 pathway, by a high sugar environment, which would increase AGEs, decreases lifespan reduces antioxidant levels and increases OS. IGF1 can reduce circulating glycation substrates and may be a mediator of OS and may also have links to telomere length. In the Cardiovascular Health Study adults over 65 years old (Kaplan *et al.*, 2009) and in a study of adults from Campina aged over 85 years old (Barbieri *et al.*, 2009) IGF1 levels in peripheral blood samples were significantly positively correlated with LTL. However, cultured human skin fibroblasts treated with IGF1 showed increased telomere shortening versus vehicle via upregulation of the p53-p21 pathway (Matsumoto *et al.*, 2015). As such some further regulatory mechanism may be involved, such that both over and under expression of IGF1 can contribute to cellular senescence and therefore ageing.

It is not solely changes in the hormone insulin, and its pathways, which impact ageing. One of the most notable system changes with age is the decline and halting of reproductive capabilities, or menopause, in females. Female rats, like humans, demonstrate a gradual decrease in serum estradiol levels which occurs from 12 months of age (Moorthy *et al.*, 2005). In humans decreasing estrogen leads to increased risk of: atherosclerosis and cardiovascular diseases, whereby pre-menopausal women

show lower incidence of cardiovascular disease than aged matched men but post-menopause have higher levels of incidence (Hayward, Kelly and Collins, 2000; Erhardt, 2003; Wake and Yoshiyama, 2009); cognitive decline as seen in women post-menopause performing significantly worse than those pre-menopausal and in late menopausal stages on cognitive assessments (Sherwin, 2009; Epperson, Sammel and Freeman, 2013; Weber, Rubin and Maki, 2013); osteoporosis, as estrogen deficiency activates osteoclasts and osteoblasts which cause decreased bone mass (Fan *et al.*, 2014; Miyamoto, 2015) and skin ageing and reduced wound healing abilities, as decreased estrogen levels are correlated with collagen loss and estrogen use post-menopause increases collagen content, thickness and elasticity of the skin (Calleja-Agius and Brincat, 2012; Horng *et al.*, 2017; Wilkinson and Hardman, 2017). In addition to estrogen *per se*, a decline in estrogen receptors α (ERα, (Marosi *et al.*, 2012)) and β (ERβ, (Munetomo *et al.*, 2015)) occurs with age in rodents and have been linked to these ageing factors. The effects of estrogen receptors on heart disease can be seen in receptor deficient or knock-out mice. Female ERβ null mice show increased mortality, heart failure and impaired calcium modulation after myocardial infarct compared to controls (Pelzer *et al.*, 2005). Moreover, mice deficient in ERβ but not ERα demonstrated prolonged ventricular repolarisation after myocardial infarct relative to both control infract mice and ERβ deficient mice without infarct (Korte *et al.*, 2005) suggesting this receptor is important in myocardial recovery.

In terms of cognition ovariectomised (OVX) ERα knock-out mice can perform normally on the Morris water maze task but, whilst controls demonstrate poorer performance on this task when treated with estrogen, knock-out mice did not. This data suggests this receptor is important in the mediation of estrogens impact on spatial memory (Kim and Casadesus, 2010). OVX ERβ knock-out mice demonstrated delayed learning on the Morris water maze at low doses of estrogen and complete inability to learn the task at high estrogen doses (Rissman *et al.*, 2002). In humans, male and female, polymorphisms in ERα and ERβ increased the risk of cognitive decline, however the exact impact on function of these changes is as yet unknown (Yaffe *et al.*, 2002, 2009). As such it is likely an imbalance of ER expression within the brain has an impact on learning with age.

Studying the effect of ERs on bone density has shown both female and male ERα knock-out mice have decreased cortical bone density and increased trabecular bone density and the ERβ knock-out led to decreased bone resorption and increased trabecular bone volume in females but not males (Sims *et al.*, 2002) demonstrating a sex difference in the control of bone health. OVX has also been used to model osteoporosis as it induces loss of cancellous bone and increases osteoclasts in mice (Jilka *et al.*, 1992). OVX mice which are ERβ deficient lose trabecular bone density more slowly than OVX controls for the first 3-5 months after treatment, suggesting a loss of ERβ was protective against bone loss in the early stages (Seidlova-Wuttke, Nguyen and Wuttke, 2012), whereas inhibition of ERα in OVX mice exacerbated bone loss (Bae *et al.*, 2017). As such age-related changes in both hormone levels and receptor expression can be seen to influence decline in function with age which is most clearly evidenced in post-menopausal females and equivalent rodent models.

Looking across the sexes, changes in gastrointestinal and feeding hormones have been linked to both normal and abnormal ageing. A common occurrence with ageing is a loss of appetite and weight or anorexia of ageing (Bhutto and Morley, 2008; Visvanathan, 2015). It is thought that this occurs due to differences in hunger and satiety sensations and a decline in ability to regulate appetite (Moss *et al.*, 2012) caused by changes in response to satiety and hunger hormones (Bauer *et al.*, 2010). Brain tissue and serum from aged Fisher rats was used to measure changes in cytokines, gastrointestinal hormones and amyloid precursor protein (APP) to study the effects of weight and ageing. Rats were fed *ad libitum* (aged 4, 12 or 20months old), calorie restricted from 4-20months old or calorie restricted until 20months old and then converted to an *ad libitum* diet by 15% food increase every 2 days over 7 days (refed). *Ad libitum* rats aged 4 and 12 months old were used as controls for age related changes. The study found that brain weight was influenced by body weight rather than ageing, as although brain weight increased from 4 to 12months in control rats, thereafter it plateaued, and this matched the pattern of change in body weight. Further across groups of 20month old rats' brain and body weight was reduced by calorie restriction, which was partly recovered in refed groups who regained weight but not to *ad libitum* levels. Within the brain leptin, glucagon and GLP-1 did not change with age but

PAI-1 decreased at 20-months, however leptin and GLP-1 decreased with calorie restriction with partial recovery in the refed group. Of the cytokines measured only erythropoietin (EPO) and macrophage colony-stimulating factor (M-CSF) decreased with age in serum whereas in the brain all cytokines except TNF decreased by 12 months of age. However, diet had no effect on the brain cytokine levels, but serum showed decreased EPO and M-CSF with calorie restriction and recovery of EPO but not M-CSF with refeeding. Refed rats also had lower levels of inflammatory markers than *ad libitum* rats. Finally, brain levels of APP were not seen to increase with age however calorie restriction increased levels and refeeding exacerbated this effect, though not significantly. Altogether this suggests that both age and nutrition can impact brain and periphery differently and that APP is more influenced by feeding hormones than either age or cytokines (Banks, Abrass and Hansen, 2016). This study along with the earlier presented evidence of the ability of caloric restriction to increase lifespan suggest that nutritional status and feeding hormones rather than ageing *per se* may have a greater influence on changes over time within the body, especially within the brain. This poses the question of the role of hormones in pathological ageing, especially neurodegenerative diseases, and in the scope of this thesis more specifically Alzheimer's disease.

3.3 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the biggest cause of dementia accounting for 60-80% of cases. Psychologically It can be characterised by a gradual decline in cognitive function which becomes sufficient to impact activities of daily living. Behaviourally this may be seen initially as difficulties with short-term memory, such as forgetting names or recent events, which gradually worsens to frequent disorientation, inability to recognise friends and family, confusion and eventually difficulty walking, speaking and swallowing. These changes are accompanied by pathology in the brain consisting of the accumulation of amyloid beta (Aβ) plaques and tau neurofibrillary tangles (NFT), cell damage and neuronal death. The biggest risk factor for developing AD is age (Alzheimer's Association, 2016; Lane, Hardy and Schott, 2018).

Aβ is a product cut from the amyloid precursor protein (APP). APP is inserted into the cell membrane and then cleaved at amino acid 16 to produce a larger secreted section and a smaller transmembrane fragment, neither of which contain the entire Aβ motif (Hardy and Higgins, 1992; Cai, Golde and Younkin, 1993). Cleavage of APP is dependent on secretase enzymes and this non-amyloidogenic pathway is mediated by α-secretase, however processing of APP can also be amyloidogenic and this is mediated by β-secretase which cuts the protein further from the membrane releasing soluble APP β. The additional cleavage of the remaining transmembrane protein by γ-secretase produces Aβ (Wilkins and Swerdlow, 2017). It is this component which is integral to plaque formation in AD. Aβ can form both fibrils and oligomers, with varying numbers of residues, both of which have been linked with disease type, progression and neurotoxicity (Sengupta, Nilson and Kayed, 2016; Qiang *et al.*, 2017). Plaques consist largely of fibrils of predominantly $A\beta_{1-42}$ (Roher *et al.*, 1993) and the treatment of neurons with soluble $A\beta_{1-42}$ causes toxicity and the formation of fibrils both at the cell body and along neurites which leads to beading and fragmentation (Krishtal *et al.*, 2017).

Tau is a microtubule associated protein which is required for microtubule assembly from tubulin (Cleveland, Hwo and Kirschner, 1977). Tau also binds to actin filaments to cross-link the cellular cytoskeleton and therefore is important for neurite outgrowth (Cabrales Fontela *et al.*, 2017) as demonstrated in non-neuronal cells where over expression of the tau protein generates long processes similar in appearance to neuronal axons (Knops *et al.*, 1991). Blocking tau in NB2a/d1 neuroblastoma cells prevents neurite elongation (Shea *et al.*, 1992). Tau can be modified post-translation, for example by phosphorylation. Phosphorylating tau at serine 262 (Ser262) decreases tau binding to actin filaments and as such can reduce this interaction (Cabrales Fontela *et al.*, 2017). In AD tau is hyperphosphorylated on at least 25 sites (Hanger *et al.*, 1998) and it polymerises into NFTs comprising of paired helical filaments and straight filaments (Grundke-Iqbal *et al.*, 1986; Crowther, 1991). These tangles aggregate normal tau hence preventing microtubule assembly and destabilising existing structures (Lindwall and Cole, 1984; Bramblett *et al.*, 1993). Dephosphorylation of hyperphosphorylated tau from AD brain cytosol inhibits the formation of paired helical and straight filaments and re-phosphorylation promotes

this pathology. Phosphorylation sites Threonine 231 (Thr231) and Ser262 were found to be essential for this action as phosphorylating agents which did not affect these sites did not result in filament self-assembly (Wang, Grundke-Iqbal and Iqbal, 2007). Studying the pattern of NFT formation in the temporal lobe of AD patient brain samples it was seen that tau phosphorylation positively correlates with neuronal loss and phosphorylation at Thr153, Ser262 and Thr175/181 was identified in the earliest stages of the disease (Augustinack *et al.*, 2002).

Whilst NFTs form initially in the entorhinal region then spread to the hippocampal area and finally the neocortex (Braak and Braak, 1991), Aβ pathology begins in the neocortex and spreads to entorhinal area, hippocampus, subcortical regions and then finally brain stem and cerebellum (Thal *et al.*, 2002). Despite understanding the pattern and progression of the disease the underlying causes remain unclear. Links have been made between the different theories of ageing and AD. In hippocampal neuronal cultures Aβ triggered increased ROS production which was blocked by inhibition of N-methyl-D-aspartate (NMDA) receptors (De Felice *et al.*, 2007) and mice lacking SOD have high levels of tau hyperphosphorylation and when crossed with the AD model Tg2576, which show brain amyloid pathology by 16 months of age, amyloid burden is increased (Melov *et al.*, 2007). This demonstrates that OS is important in the exacerbation of the disease. Some data suggests that mitochondrial dysfunction could precede Aβ pathology as the triple-transgenic AD female mouse model shows decreased mitochondrial function and increased OS from 3 months of age but Aβ did not increase until 9 months. Mitochondrial dysfunction was further exacerbated by reproductive senescence at 12months old (Yao *et al.*, 2009). A theory known as the Inverse Warburg hypothesis suggests that AD is initiated by age-related mitochondrial decline whereby oxidative phosphorylation and ROS production are increased in some neurons, possibly due to a decline in energy-producing abilities, which will compete with healthy neurons for energy sources, e.g. glucose, depleting levels nearby causing neighbouring neurons to also try to increase energy production in the same manner or induce cell death (Demetrius and Simon, 2012; Demetrius and Driver, 2013; Demetrius, Magistretti and Pellerin, 2015;

Grimm, Friedland and Eckert, 2016). However, this theory requires further exploration as to why this would occur and lead to AD in some ageing adults and not others.

The relationship between AD and telomeres is unclear. Whilst telomerase activity is seen to be increased in lymphocytes of AD patients and telomerase activity in lymphocytes was correlated with AD severity (Zhang *et al.*, 2003), LTL length in AD patients cerebrospinal fluid (CSF) was similar to healthy controls (Movérare-Skrtic *et al.*, 2012) and telomere shortening in the APP23 AD mouse model reduced Aβ plaques and microgliosis (Rolyan *et al.*, 2011). However, Aβ has been shown to inhibit telomerase activity and increase SA-β-gal in PC12 and HeLa cells (J. Wang *et al.*, 2015), suggesting that telomere changes may be involved in the pathology of AD as opposed to its initiation.

Glycation and AGE's have also been implicated in AD. Immunohistochemical analysis of Aβ, tau and AGEs in brain tissue from AD patients show all Aβ plaques and intracellular NFTs contained AGEs (Sasaki *et al.*, 1998). The receptor for AGEs (RAGE) also has a role in AD, as Aβ can interact with RAGE both to cross the blood-brain barrier (BBB) and to disrupt its integrity by inducing OS and inflammation (Cai *et al.*, 2016). Cortical mouse neurons when treated with human Aβ showed intracellular uptake to the cytosol and mitochondria, which was significantly reduced by RAGE inhibition and were protected from Aβ-induced mitochondrial dysfunction. This demonstrates an important role in the transport of Aβ and its impacts on the mitochondria (Takuma *et al.*, 2009). AGE signalling through RAGE has been shown to increase cathepsin B mediated APP processing, leading to increased Aβ and increased tau phosphorylation in primary cortical neurons. Further temporal cortex samples from AD patients showed increased RAGE, cathepsin B and asparagines endopeptidase, correlate with increased AGE modified proteins and phosphorylated tau levels (Batkulwar *et al.*, 2018). Whilst this may suggest a role for AGE and RAGE signalling in the initiation of AD the nature of this relationship is unclear. The triple transgenic mouse model of AD found no significant changes in RAGE at 10 months but increases in expression in the hippocampus and cortex were seen in 22-24month old mice compared to age-matched controls. Expression of RAGE co-localised with intracellular APP and Aβ, and tau in the

hippocampal CA1 area (Choi *et al.*, 2014). As these mice show Aβ increases from 9 months (Yao *et al.*, 2009) this would suggest that Aβ pathology precedes changes in RAGE and hence increased RAGE expression is a result of AD pathology rather than a cause and helps to further exacerbate pathology. Interestingly a diet high in AGE containing foods in the Tg2576 AD mouse model led to significantly reduced spatial memory, higher hippocampal levels of Aβ and AGEs, greater vascular levels of OS and increased expression of RAGE (Lubitz *et al.*, 2016). As such this indicates a role for diet in AD, or at least in exacerbating its pathology.

Other than ageing two major risk factors for AD are obesity and diabetes. Obesity significantly increases cognitive decline in ageing (Tucsek *et al.*, 2014) and exacerbates pathologies (Leboucher *et al.*, 2013) in AD. In aged mice given a high-fat diet, inflammation and BBB disruption induced by obesity were exacerbated with age, leading to microglial activation, upregulation of proinflammatory cytokines and oxidative stress. Further, microglia grown in culture when exposed to sera from these mice showed pronounced activation and oxidative stress (Tucsek *et al.*, 2014). This shows how the combination of age and obesity can increase detrimental effects of ageing alone. The effects of high-fat diet in AD mouse models have been explored. In the APP/PSN1 transgenic mouse model high-fat diet induced higher triglyceride concentrations and insulin intolerance than wild type controls (Petrov *et al.*, 2015). Further, these mice displayed increased memory impairment, Aβ oligomers and deposition, which could be improved through exercise (Maesako *et al.*, 2012).

Individuals with type 2 diabetes show accelerated cognitive decline relative to aged-matched individuals without diabetes (Spauwen *et al.*, 2013) and demonstrated greater risk of developing dementia (Cukierman, Gerstein and Williamson, 2005). Further insulin resistance alone was seen to produce a pattern of reduced glucose metabolic rate in the brain similar to that of AD patients and poorer performance on a memory task compared to healthy controls (Baker *et al.*, 2011). Diet induced insulin resistance in the Tg2576 mice increased Aβ in the brain and promoted amyloidogenic processing of APP (Ho *et al.*, 2004) and in patient studies insulin resistance was associated with higher

phosphorylated and total tau levels in CSF (Starks *et al.*, 2015; Hoscheidt *et al.*, 2016). Together these demonstrate how diabetes can exacerbate AD but also how insulin impairment can generate AD pathologies and precede AD onset. A theory has even been posited that AD is simply a new type of diabetes, type-3 diabetes, however this theory is largely based on work in animal models (Kandimalla, Thirumala and Reddy, 2017) and Phase III trials for an insulin-sensitising drug, pioglitazone, as a treatment in AD have recently been terminated due to lack of efficacy (identification no. NCT01931566 and NCT02284906).

Despite this, both obesity and diabetes are diseases for which a causative role of hormone insensitivity has been identified. Given this and the evidence presented that shows the important role of diet in ageing and specifically how the brain can be differentially affected by this than the periphery, this suggests that alterations in satiety related hormones may provide interesting new avenues for treatments. Two such hormones which have been linked with ageing are leptin (Folch *et al.*, 2012; Gulcelik *et al.*, 2013; Balaskó *et al.*, 2014) and cholecystokinin (CCK) (MacIntosh *et al.*, 2001; Sun, Lu and Cai, 2005; Moss *et al.*, 2012). However, the potential for these molecules within normal and abnormal ageing have not yet been fully explored. Within this thesis I aim to demonstrate a novel view of these two adipokines and their potential uses within pathological ageing.

3.4 LEPTIN

Leptin is an 167 amino acid hormone encoded by the ob gene, which regulates satiety and energy homeostasis via the hypothalamus (Zhang *et al.*, 1994; Mandal, Prabhavalkar and Bhatt, 2018). Leptin is primarily produced by adipocytes (Myers and Greenwald-Yarnell, 2013) the most abundant cell type within white fat adipose tissue (Martinez-Santibañez, Cho and Lumeng, 2014). Leptin release is regulated by food intake. Increases in triglyceride stores leads to increased leptin production whereas decreased fat stores lead to decreased leptin production. As such, leptin is a long-term modulator of total body fat and hence weight gain and loss (Friedman and Halaas, 1998). This is evidenced in the correlating high or low circulating leptin levels identified in individuals with high or low adipose levels

respectively (Page-Wilson *et al.*, 2015). Hence it follows that high fat stores should cause an individual to eat less to maintain a healthy adipose level (**Figure 2**). Leptin is also expressed in the brain. Leptin expression has been identified in pituitary, cerebellum, cerebral cortex and hypothalamus (Morash *et al.*, 1999).

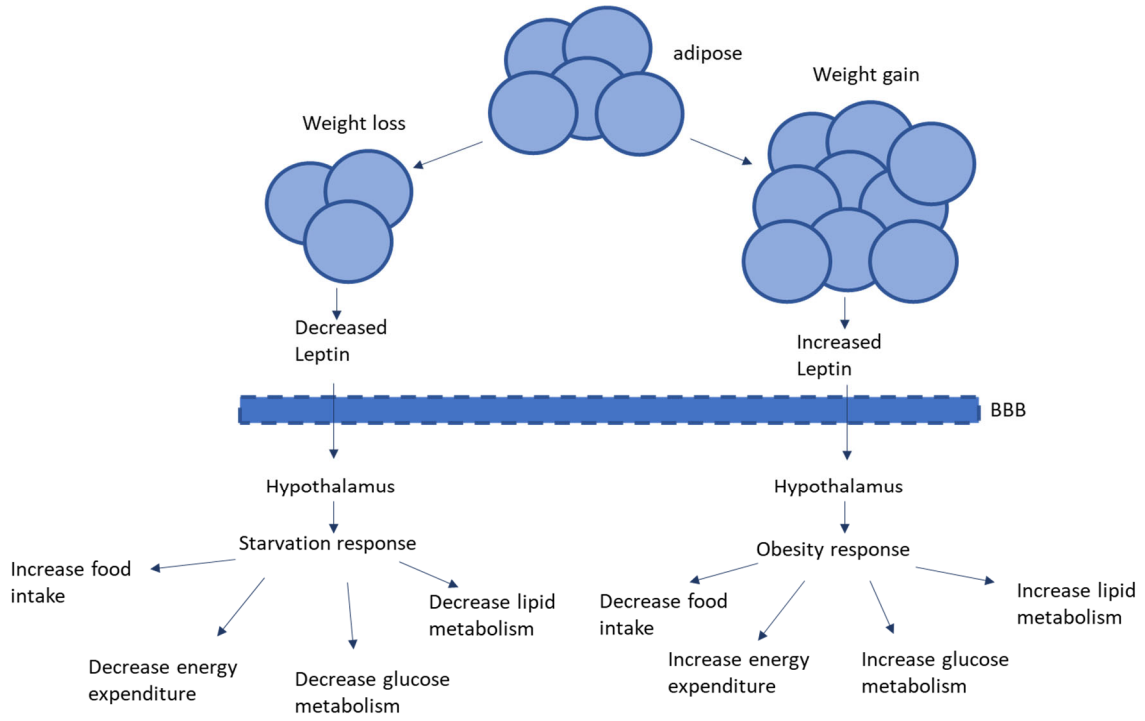

Figure 2. Effect of adipose tissue on leptin signalling. Weight loss leads to a decrease in adipose tissue and therefore a decrease in leptin levels. Less leptin crosses the blood-brain barrier (BBB) to reach the hypothalamus and hence signals to decrease energy expenditure and metabolism and increase food intake by generating a sensation of hunger. Weight gain causes the opposite effect whereby increased adipose tissue leads to increased leptin leading to decreased food intake and increased energy expenditure and metabolism (Friedman and Halaas, 1998).

Leptin has six different receptor isotypes; four of these are short form (ObRa, ObRc, ObRd and ObRf), one is long (ObRb) and one is soluble (ObRe) (Lee *et al.*, 1996). These receptors are predominantly membrane-associated but Ob-Re is a secreted form of the receptor and consists of only an extracellular domain (**Figure 3**). In plasma leptin can be found both unbound and bound to other proteins, one of which is ObRe (Li *et al.*, 1998). As such ObRe modulates circulating leptin levels by binding to it and delaying its re-uptake. This is evidenced in Zucker rats which show a 20-fold increase in ObRe under high fat conditions, overexpression of the receptor leads to a similar increase in

circulating levels and in ob/ob mice which lack leptin, exogenously administered leptin has higher detection when ObRe is overexpressed (Huang, Wang and Li, 2001). Evidence has also been seen in humans where it was seen that leptin levels were correlated with ObRe levels and after a 72h fast leptin levels decreased 80% but ObRe increased 100% (Chan *et al.*, 2002). So ObRe is likely to play an important role in metabolic disorders, such as obesity where even though leptin levels are increased due to greater adipose tissue ObRe levels decrease compared to healthy controls (Chan *et al.*, 2002; Reinehr *et al.*, 2005) and diabetes where ObRe levels have been seen to increase in children with type 1 diabetes (Kratzsch *et al.*, 2004, 2006), and can impact sensitivity to the leptin hormone by decreasing its availability (Schaab and Kratzsch, 2015).

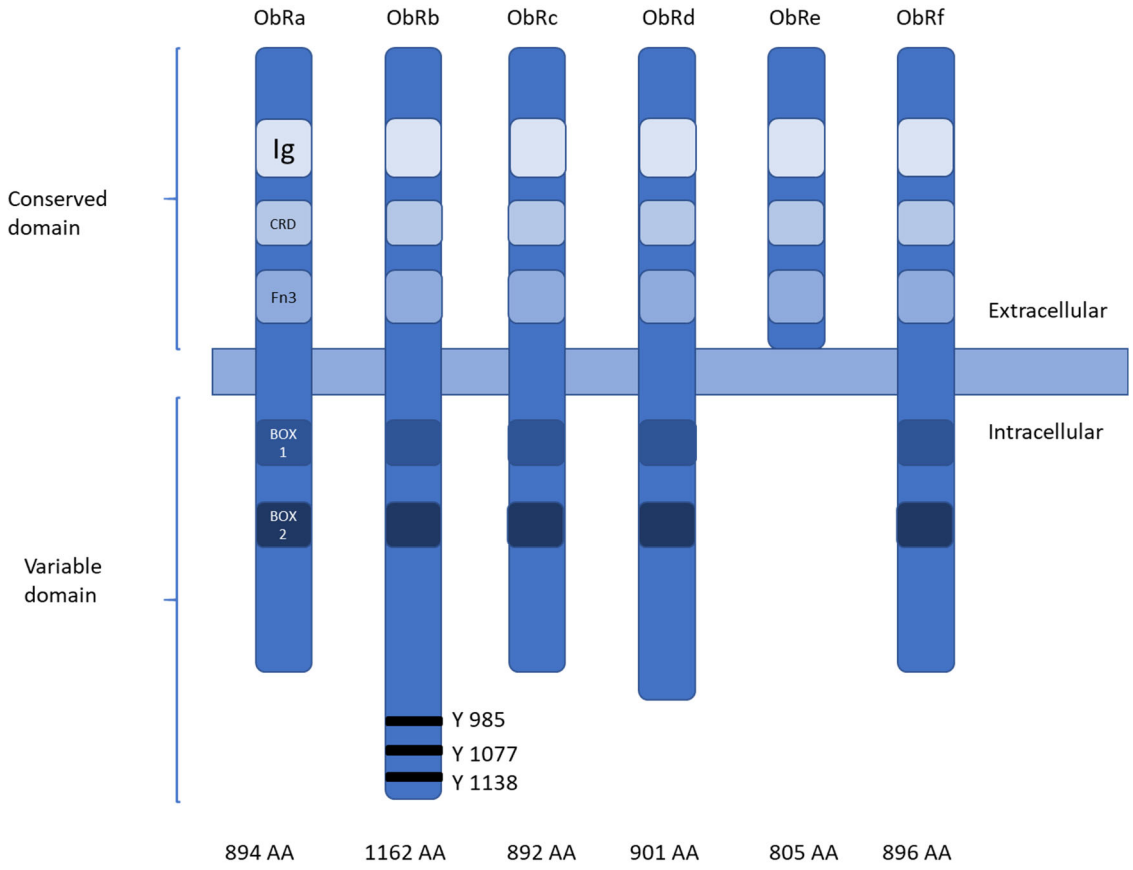

Figure 3. Leptin receptor isoforms. All isoforms of the leptin receptor have the same conserved extracellular domain, which contains an immunoglobulin-like domain (Ig) and cytokine receptor homologous domain (CRD), where leptin binds, and fibronectin type 3 domain (Fn3) needed for receptor activation. In the intracellular domain BOX 1 and BOX 2 are required for leptins JAK signalling. In ObRb additional tyrosine residues (Y 985, 1077 and 1138) may be considered a BOX 3 motif which is essential for STAT3 signalling (Bjørbaek *et al.*, 1997; Zabeau *et al.*, 2005; Prokop *et al.*, 2012; Wauman, Zabeau and Tavernier, 2017).

The remaining receptor isoforms; ObRa, ObRb, ObRc, ObRd and ObRf are all transmembrane proteins. These all have similar extracellular components (as does ObRe) but differ in their intracellular segments. The short isoforms have intracellular domains of 32-40 amino acids and are identical for the first 29 of these, which contains a BOX 1 and 2 motifs and JAK tyrosine kinase (Gorska *et al.*, 2010). The function of these short-form receptors is poorly understood and expression of isoforms ObRc, ObRd and ObRf are extremely low, although due to their BOX motifs are assumed to have some signalling capabilities (Fei *et al.*, 1997; Belouzard and Rouillé, 2006; Séron *et al.*, 2011). ObRc and ObRf have been shown to be the most highly expressed isoforms in the rat carotid body (Porzionato *et al.*, 2011) and increased in prostate and testis of food restricted rats (Gombar and Ramos, 2013) but the significance of these findings was not discussed. When ObRc was expressed in *Xenopus laevis* oocytes leptin binding and internalisation was observed and co-expression of human sodium/glucose cotransporter demonstrated that leptin increased sugar uptake into oocytes suggesting ObRc is able to mediate leptin internalisation (Barrenetxe *et al.*, 2003). Further HEK293 cells overexpressing ObRa, ObRb, ObRc or ObRd could all bind and endocytose leptin and once it was internalised degradation and exocytosis was independent of receptor isoform (Tu *et al.*, 2007) as such the significance of these isoforms remains to be explored.

ObRa is the most predominant form in peripheral tissues and has some reduced leptin-mediated signalling function as it undergoes leptin-dependent phosphorylation of JAK2 which disappears when its BOX 1 motif is mutated and in CHO cells expressing ObRa leptin stimulated ERK2 phosphorylation (Bjørbaek *et al.*, 1997; Löllmann *et al.*, 1997). It is also the most prevalent leptin receptor in the BBB and is thought to allow leptin access to receptors throughout the brain (Boado *et al.*, 1998; Harvey *et al.*, 2005). This mechanism is saturable (Banks *et al.*, 1996), hence past a critical level of leptin, increasing blood levels will have little impact and in agreement with this obese rats, with high circulating blood levels of leptin, have no significant increase in brain levels of leptin compared to controls (B Burguera *et al.*, 2000). However, it has also been shown that rats fed a high-fat diet showed increased expression of the Ob-Ra receptor in the BBB (Boado *et al.*, 1998), which would be expected

to compensate for the saturability of the leptin receptors. Conversely, a more recent study found that inhibiting ObR in an in vitro human endothelial cell line (a model of the BBB) did not prevent leptin transport, and suggested that the low-density lipoprotein receptor-related protein-2 (LRP-2) may be a more suitable candidate for leptin transport across the BBB, as these cells express LRP-2 (Gonzalez-Carter *et al.*, 2016), it has been shown to bind leptin at the blood-CSF barrier and blocking this receptor reduced leptin crossing into CSF (Dietrich *et al.*, 2008).

Within the brain ObRb is the most highly expressed form of the receptor. It consists of a long, 303 amino acid, intracellular domain which contains BOX 3 and SOCS motifs. It is considered the fully active form and is mainly expressed in the hypothalamus (Gorska *et al.*, 2010). ObRb is expressed in other areas of the brain including in abundance in the hippocampus which is important in learning and memory (Harvey *et al.*, 2005; McGregor and Harvey, 2017). The link between leptin and learning and memory has been widely reported. Leptin deficiency has been induced in rodents using both high-fat diets and leptin receptor deficient models (Sharieh Hosseini, Khatamsaz and Shariati, 2014; Mandal, Prabhavalkar and Bhatt, 2018). Both of these models have demonstrated impaired spatial memory performance on the Morris water maze paradigm (X-L Li *et al.*, 2002; Yu *et al.*, 2010; Valladolid-Acebes *et al.*, 2013; Sharieh Hosseini, Khatamsaz and Shariati, 2014; Cordner and Tamashiro, 2015). Further mouse db/db models with leptin receptor deficiency showed impaired extinction of conditioned taste aversion (Ohta *et al.*, 2003), whereas bilateral injections of leptin into healthy mice following training on T-maze shock avoidance and one-trial step down inhibitory avoidance tests improved memory consolidation compared to controls (Farr, Banks and Morley, 2006). Additionally subcutaneous leptin injections in diabetic rats demonstrated improved spatial memory (Ghasemi *et al.*, 2016).

Leptin impaired and receptor deficient models also both show changes in the hippocampus. Recordings from CA1 hippocampal slices demonstrated impaired long term potentiation (LTP) and long term depression (LTD), which are required for learning and memory formation, and lower basal Ca2+/calmodulin-dependent protein kinase II activity, which is part of the underlying signal modulating

LTP and LTD (X-L Li *et al.*, 2002). Further, mice on a high fat diet from adolescence developed leptin resistance, learning and memory deficiency and in the CA1 of the hippocampus had increased dendritic spine density, up-regulation of neural cell adhesion molecule and desensitisation of the protein kinase B (Akt) pathway. Whilst increasing spine density and neural cell adhesion molecule are usually associated with improved learning and memory it was hypothesised that this was a compensatory mechanism to try and overcome deficits caused by leptin insensitivity. Desensitisation of the Akt pathway demonstrates impaired leptin signalling, suggesting this is the cause the memory impairment in these animals (Figure 4) (Valladolid-Acebes *et al.*, 2013).

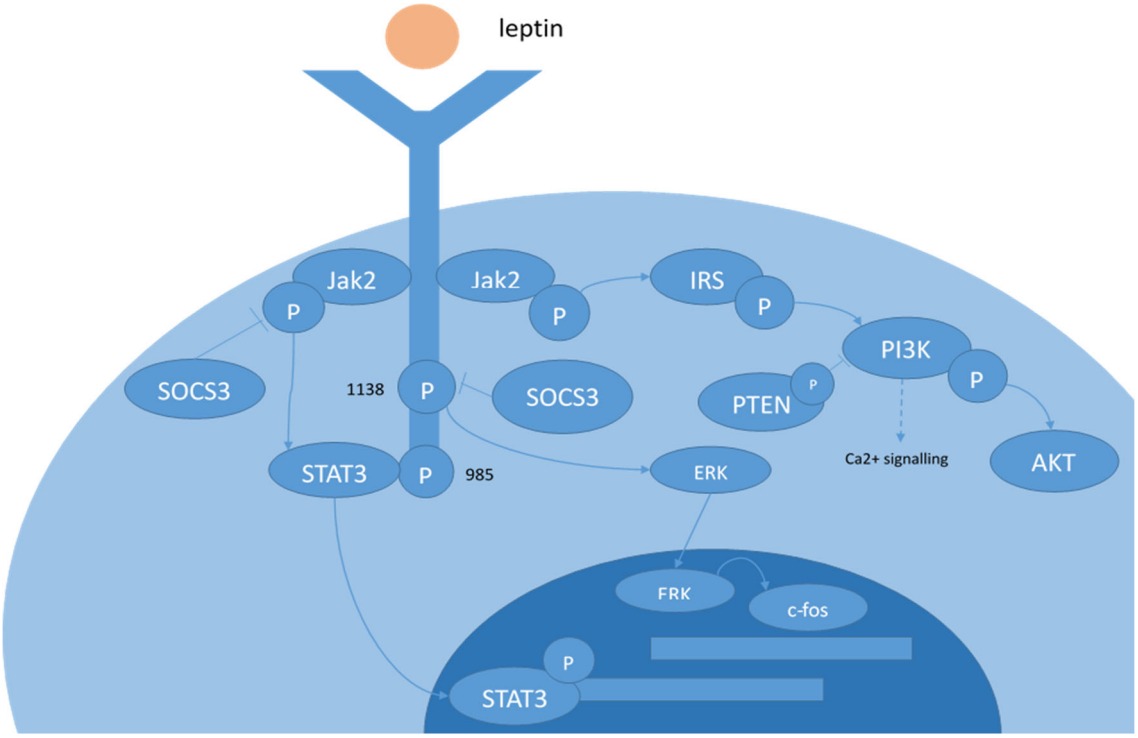

Figure 4. Downstream signalling pathways of the leptin molecule once bound to ObRb receptor. STAT3 and Akt are cell survival pathways lending weight to leptin as a neuroprotective molecule. Jak = Janus kinase, SOCS = suppressor of cytokine signalling, STAT = signal transducer and activator of transcription proteins, ERK = extracellular signal-related kinase, c-fos = Fos proto-oncogene, IRS = insulin receptor substrate, PI3K = phosphatidylinositol 3-kinase, AKT = protein kinase B, PTEN = phosphate and tensin homolog.

In human obesity, this decreased sensitivity of leptin receptors in the hippocampus means individuals may display impaired learning and memory capabilities, as suggested by rodent models

where high fat diets induce leptin resistance and deficits in hippocampal reliant behaviours (Kanoski *et al.*, 2007; Granholm *et al.*, 2008; Boitard *et al.*, 2014). Data from the Framingham study demonstrates that men with obesity have impaired cognitive performance, which was further decreased by the presence of hypertension (Elias *et al.*, 2003). Further obese individuals have an impaired ability to learn avoidance of negative outcomes (Coppin *et al.*, 2014). This association is not limited to adults, as children classified as overweight or obese had a negative association between abdominal fat deposition and hippocampal-dependent relational memory (Khan *et al.*, 2015). However, individuals who had undergone bariatric surgery have improved memory performance associated with their decreased BMI (Alosco *et al.*, 2014).

Leptin resistance has been associated with ageing. In rats modelling old age leptin levels in young rats by subcutaneous leptin infusion led to decreased food intake and visceral fat levels and increased insulin functioning compared to controls. In the old rats increasing leptin levels had no impact on food intake, fat or insulin, suggesting resistance or impaired leptin response (Ma *et al.*, 2002). In humans testing leptin resistance with age directly is difficult and whilst some studies suggest serum leptin levels increase with age (Roszkowska-Gancarz *et al.*, 2015), when BMI is accounted for data suggests there is a decrease (Isidori *et al.*, 2000; Baranowska *et al.*, 2007). Other aspects of the leptin system are affected by age however, ObRa, but not ObRb, was found to have decreased expression in elderly non-obese adults compared to young adults (Roszkowska-Gancarz *et al.*, 2015) which would reduce responsiveness to leptin with age. Leptins also has links to AD. Plasma leptin concentrations were measured from dementia-free participants in the Framingham Original cohort and levels were rechecked and MRI's for brain volume taken at follow-up testing. From the cohort of 198 participants, 111 developed dementia, 89 of which were diagnosed with AD. It was seen that patients with higher leptin levels had a reduced risk of dementia and AD and greater cerebral volume, suggesting that leptin levels are directly related to brain health (Lieb *et al.*, 2009). Further this trend was confirmed in elderly women, where higher serum leptin was associated with lower incidence of dementia and MCI with normal BMI. However in overweight and obese women this trend was not seen, which suggests the

leptin-resistance associated with obesity prevents the protective benefits of increased leptin levels (Zeki Al Hazzouri *et al.*, 2013).

The effects of leptin in AD have been further explored in animal and cell models. The APP/PS1 transgenic mouse model display elevated hippocampal Aβ and memory loss from 6 months old. Studying changes to the leptin system in these mice demonstrates reduced ObR expression at 6 months old and decrease in SOCS, a downstream molecule of the leptin signalling pathway, from 3 months old (Pedrós *et al.*, 2015). Treatment of these mice with leptin shows an increase in proliferation of neuronal precursors and attenuates Aβ-induced neurodegeneration, suggesting leptin can have both neurotrophic and neuroprotective effects (Pérez-González *et al.*, 2011). In the TgCRND8 AD model 8 weeks of leptin treatment reduced Aβ in brain and serum and decreased p-tau, as well as improved performance on memory tests (Greco *et al.*, 2010). In cell models induced tau phosphorylation was seen be significantly reduced by leptin in SHSY-5Y, NT2 and rat primary cortical neurons (Greco *et al.*, 2008). In hippocampal slices exposed to Aβ LTP is blocked and LTD is facilitated, an effect which is reversed by leptin and seen to be dependent on the PI3K signalling pathway as inhibition of this prevented leptins effects on LTD and abilities to prevent AMPA receptor internalisation. Leptin also protected primary cortical neuron cultures from Aβ induced cell death and this action was seen to be dependent on the STAT3 pathway as leptin alone increased phosphorylation of STAT3 and inhibition of STAT3 in cultures treated with leptin and Aβ negated its protective effects (Doherty *et al.*, 2013). Together this data demonstrates that leptin could have beneficial disease-altering effects as a treatment for AD.

However, whilst data suggests that leptin has merit as a potential therapeutic in AD there are issues surrounding use of the full-length molecule. As described, there are many different isoforms of the leptin receptor, most with some degree of signalling capabilities and these are located throughout the body. Further to fat tissue and the brain leptin receptors have also been located in the digestive system (Breidert *et al.*, 1999), vasculature (Trovati *et al.*, 2014; Lanier *et al.*, 2016) and adrenal and

pituitary glands (Glasow *et al.*, 1999; Jin *et al.*, 1999). This demonstrates that leptin has a role across multiple systems and as such leptin as a drug would likely have widespread side effects. Leptin has been associated with endometriosis and greater sensitivity to pain. In patients diagnosed with fibromyalgia daily fluctuations in serum leptin were positively correlated with perceived pain and in healthy post-menopausal women, both increased leptin levels and BMI were independently associated with greater self-reported pain (Younger *et al.*, 2016). In a rodent model of endometrosis leptin was increased in endometriosis-like lesions. Leptin injected into the muscles of healthy rats caused increased sensitivity to pain and nociceptor sensitisation, which was further increased by ovariectomy, suggesting that estrogen may also play a role (Alvarez *et al.*, 2014). Several studies have also linked leptin to breast cancer. This link was first acknowledged after it was seen that obesity was a major risk factor for breast cancer in post-menopausal women (García-Robles, Segura-Ortega and Fafutis-Morris, 2013). In patients recently diagnosed with breast cancer it was seen that leptin, leptin corrected for BMI and free leptin index were increased and ObRe was decreased compared to matched healthy controls (Rodrigo *et al.*, 2017). In MCF-7, T47D and BT474 breast cancer cells, leptin treatment induced proliferation and upregulated gene expression of acyl-CoA cholesterolacyltransferase 2 which, has been identified as a therapeutic target in other cancers, in turn activated ObR (Huang *et al.*, 2017). Together this evidence suggests there may be serious side effects with a leptin treatment. In fact, Metreleptin, a previously FDA-approved leptin based treatment, has recently received a black box warning due to its risk of causing lymphoma and causing the body to produce anti-metreleptin antibodies which neutralise endogenous leptin activity (FDA, 2014; Diker-Cohen *et al.*, 2015).

It may be possible to adapt leptin so that it may still be useful as a therapeutic but without the negative side effects. The structure of leptin consists of four anti-parallel α-helices labelled A, B, C and D, connected by two longer cross over loops between A and B (AB loop) and C and D (CD loop) and one shorter loop between B and C (BC loop). The CD loop also contains a small helical segment named E (**Figure 5**)(Zhang *et al.*, 1997).

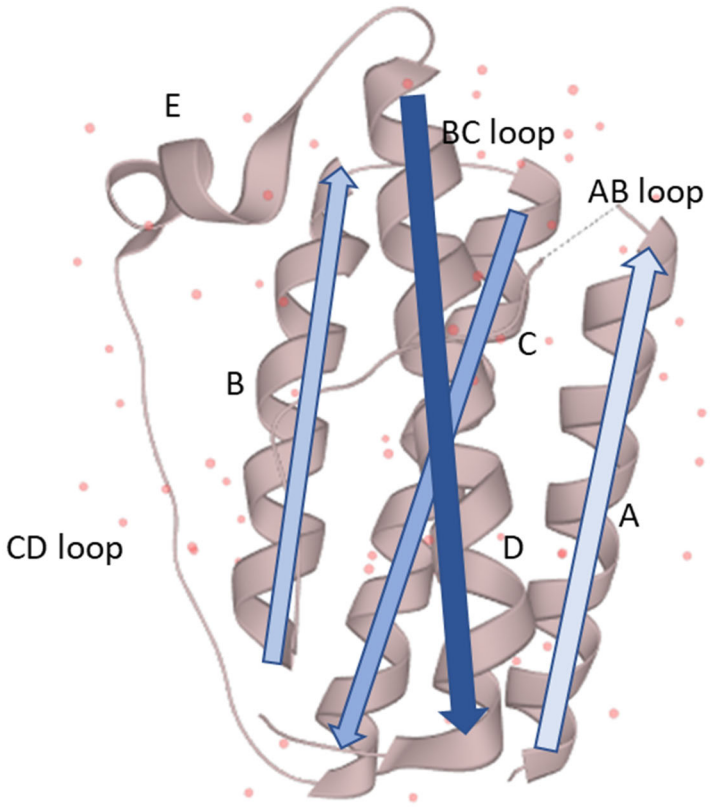

Figure 5. 3D structure of leptin. Leptin is composed of four α-helices (A, B, C and D) linked by three loops (AB, BC and CD). The CD loop contains a short helical section (E). (Zhang *et al.*, 1997; The Uniprot Consortium, 2019).

The discovery that a single base mutation in the ob gene at codon 105, so that an arginine is replaced by a stop codon, results in an inactive form of leptin (Weigle *et al.*, 1995) led to the hypothesis that leptins activity may be caused by the region distal to amino acid 105. Synthesis of small fragments of the leptin hormone from the region beyond amino acid 105 were assessed for their abilities to regulate body weight and food intake in obese ob/ob mice. 8-9-week-old C57BL/6J ob/ob mice were injected daily for 28 days with one of six leptin fragments of amino acids: 106-120, 116-130, 126-140, 136-150, 146-160 or 156-167. Leptin fragments 106-120, 116-130 and 126-140 were seen to significantly reduce weight and food intake in mice and the effect was most pronounced within the first 7 days. Mice receiving fragments 136-150, 146-160 and 156-167 gained weight but this was not significantly different from vehicle injected controls. Moreover, whilst mice receiving fragments 106-

120 or 126-140 were only 1.8% and 4.2% heavier after 28 days, respectively, compared to the 14.7% increase in vehicle controls, mice receiving fragment 116-130 were 3.4% lighter than their initial weight. No apparent toxic side effects were observed in this study. This shows that the satiety related functioning of leptin is dependent on amino acids between residues 106-140 and that fragments of the leptin hormone can produce *in vivo* effects which reflect that of full length leptin (Grasso *et al.*, 1997). This suggests that just a fragment of the leptin molecule could be used to generate its beneficial effects which may reduce extraneous side effects. Further work has focussed on fragment 116-130 as this was the most potent in the initial study. Further exploration of smaller fragments from within 116-130 demonstrated that mice receiving fragment 116-130 and 116-122 had significantly reduced food intake. Mice receiving fragments 116-120 and 116-121 consumed slightly less than vehicle controls though this difference was not significant, and mice receiving fragment 116-123 or 116-124 had similar levels of food intake to fragment 116-122. In a comparison of body weights mice receiving 116-130 saw a 12.2% decrease in weight compared to vehicles seeing a 12.5% increase, whereas mice receiving 116-122 demonstrated neither an increase or decrease in weight after 7 days (Rozhavskaya-Arena *et al.*, 2000). As such whilst changes in food intake can be affected by a smaller fragment the weight reducing effects of leptin appear to require amino acids 116-130. These amino acids are located in the flexible CD loop of leptin which has previously been described as part of leptins binding site III. Mutations in this region have shown decreased enzyme activity and inability to activate the CRD, although binding remained normal (Peelman *et al.*, 2004).

Given that leptin has been proven to have beneficial effects for neuronal health and in AD models and further that amino acids 116-130 have been identified as being essential for the hormones anti-obesity action. I aim to explore the abilities of leptin fragment 116-130 ($leptin_{116\text{-}130}$) as a neuroprotective agent and compare its abilities to that of full-length leptin in order to show that just a fragment of the hormone could produce beneficial effects in AD, with the potential of reducing off-target effects.

3.5 CHOLECYSTOKININ

Cholecystokinin (CCK) is a hormone of the adipokine family, synthesised in the small intestine by I-cells (Polak *et al.*, 1975) where it performs satiety-related functions such as reducing food-intake, inhibiting gastric emptying and gallbladder contraction (Sayegh, 2013). In the brain it is synthesised by neurons in the hypothalamus, cortex, hippocampus, amygdala, olfactory bulb and nucleus tractus solitarii (Innis *et al.*, 1979; Vanderhaeghen *et al.*, 1980). Several bioactive forms of CCK are produced by humans of differing lengths but all with the same C-terminus (-Tyr(SO_4)-Met-Gly-Trp-Met-Asp-Phe-NH_2) (Rehfeld, 2004), the most common forms of which are: CCK-8, CCK-22, CCK-33, CCK-39 and CCK-58 (Overduin *et al.*, 2014) (**Figure 6**). CCK-8 is the short molecular form and so has been used in many studies to investigate the effects of CCK on satiety (Ritter, 2004; Blevins *et al.*, 2009; Gibbons *et al.*, 2016). However, CCK-58 has been identified as the major form produced by the I-cells of rats, dogs and potentially humans and increases satiety over and above CCK-8 (Goebel-Stengel *et al.*, 2012; Overduin *et al.*, 2014; Sayegh *et al.*, 2014). Whereas CCK-8 is the more dominant form in the CNS, produced directly by synaptosomes from the cerebral cortex (Dodd, Edwardson and Dockray, 1980; Emson, Lee and Rehfeld, 1980).

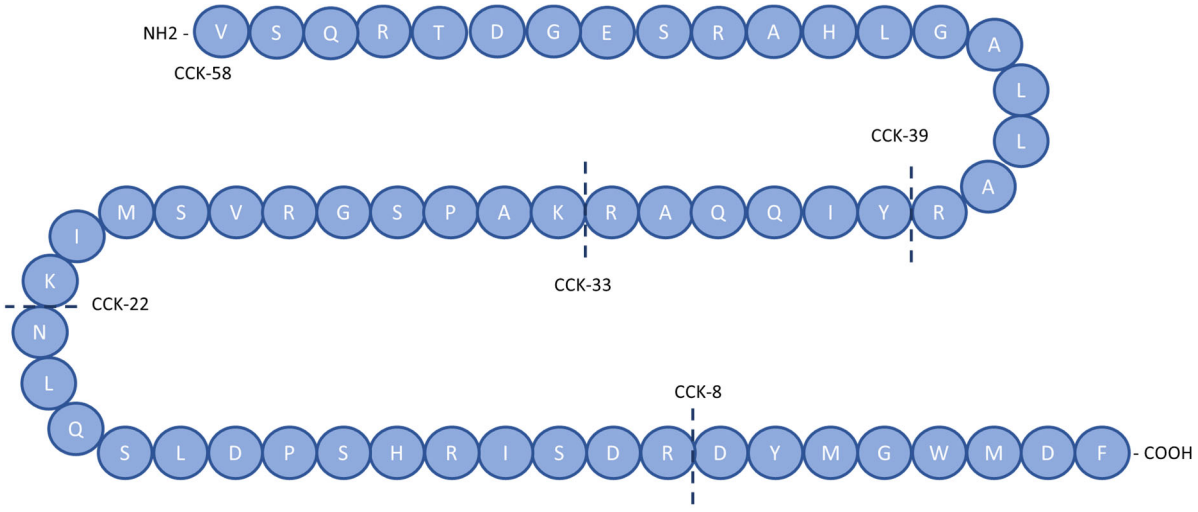

Figure 6. Structure of cholecystokinin (CCK) and its peptides. Amino acid sequence of CCK with dotted lines demonstrating where the most frequent forms would be cut CCK-8, CCK-22, CCK-33, CCK-39. CCK can also be modified post-translationally by sulfation of tyrosine at position 7 from the COOH terminal and amidation of the C-terminus (COOH becomes $CONH_2$) (Dufresne, Seva and Fourmy, 2006).

CCK acts via two receptors: CCK1R and CCK2R (Pirke *et al.*, 1994). Whilst these were originally named CCKAR (for alimentary) and CCKBR (for brain) as understanding of the receptors functioning and locations increased they were renamed CCK1R and CCK2R respectively (Dufresne, Seva and Fourmy, 2006). The two receptors have low sequence homology, but both contain 7 hydrophobic segments thought to be their transmembrane domains, with extracellular NH_2-terminal and intracellular COOH-terminal ends, indicative of the G-protein coupled receptor (GPCR) nature (Wank, 1998; Noble *et al.*, 1999; Dufresne, Seva and Fourmy, 2006) (**Figure 7**). Whilst CCK1R preferentially binds to sulphated CCK (500-1000x higher affinity (Silvente-Poirot *et al.*, 1993)), CCK2R has similar affinity for sulphated and non-sulphated forms and binds to gastrin almost as well as CCK (Straus *et al.*, 1979). Of the naturally occurring ligands CCK-8 has the highest affinity for both receptors (Ren *et al.*, 2003) but CCK1R responds readily to the other common forms e.g. CCK-33 and CCK-58; whereas CCK2R favours sulphated gastrin-17 . Roles for both receptors have been identified in: stomach in the control of acid secretion by gastrin; pancreas in the secretion of enzymes and hormones; gastrointestinal smooth muscles in secretion, growth, differentiation and motility; adipocytes in the regulation of leptin expression; adrenal gland in the secretion of aldosterone; blood mononuclear cells where it is hypothesised CCK may have antiproliferative effects although more research is needed; kidney controlling changes in potassium and sodium; and vagal afferent fibres which is suggested to be part of its satiety control function (Dufresne, Seva and Fourmy, 2006).

In terms of CNS-related function CCK2R has been well studied due to its higher abundance in the brain. CCK2R is important in the regulation of feeding and energy homeostasis as demonstrated in CCK2R deficient mice which had increased body temperature, weight and water consumption and decreased nocturnal locomotor activity (Weiland, Voudouris and Kent, 2004). This data also suggests an effect of CCK2R on movement behaviours, this is supported by work showing that rats lacking CCK1R with high circulating levels of CCK demonstrated hypolocomotion (Schroeder and Weller, 2010). However, this may also be linked to CCK2R's role in anxiety behaviours. In an unfamiliar environment stressors and fearfulness reduce locomotor activity in rodents (Rotzinger and Vaccarino, 2003).

Injection of CCK-4, which can activate both CCK1R and CCK2R, induces anxiety-like behaviours in mice. CCK2R antagonist, Cl-988, completely suppresses anxiety behaviours and changes in EEG recordings in these mice, whereas CCK1R antagonist, devazepide, was only partly able to suppress these effects (Li *et al.*, 2013). This also demonstrates a role for CCK1R in anxiety, though not to the same extent as CCK2R, but it's other CNS-related functions are less well studied. However, CCK1R does play a role in the brain. Studies have indicated: there is a CCK1R dependent neural pathway in the motor cortex which is important in glucose regulation (Little *et al.*, 2014); the presence of CCK1R mRNA in rat neuroblasts (Langmesser *et al.*, 2007); the CCK1R antagonist dexloxiglumide attenuated lipid-induced blood-oxygen level (BOLD) signalling, a measure of brain region activity, in brain stem, hypothalamus, cerebellum and motor cortex (Lassman *et al.*, 2010); and female mice lacking CCK1R demonstrated reduced neurogenesis and reduced dopaminergic neurons in the olfactory bulb (Sui *et al.*, 2013). Altogether this data suggests that CCK1R signalling could be important in neuronal cell production and growth, controlling lipid activation of several brain regions and have a role in motor cortex activities, thus suggesting CCK1R is important within the CNS. Further CCK1R may be involved in learning and memory.

OLETF rats lack CCK1R hence are a useful tool in the study of its actions. OLETF rats made significantly more errors in the radial arm maze and had lower LTP spike, determined from measurements in the hippocampus, than controls, suggesting impaired memory. However, histological comparisons of the hippocampus showed no significant differences in width or area, suggesting no significant cell loss in this area. It was concluded that these results indicate a reduction in plasticity in the dentate gyrus granular cells, which may be related to the impaired memory function. OLEFT rats are typically obese, although this was controlled for in this study by food restriction. These rats also develop diabetes by ~18 weeks of age, however, in this experiment the rats were only 13 weeks old and no urinary glucose was detected, eliminating the possibility of effects due to diabetes (Nomoto et al. 1999). OLETF rats also made significantly more errors and less correct choices than control rats in the Morris water maze, with no increase in glucose blood levels in OLEFT rats compared to controls demonstrating effects were not due to diabetes (Matsushita et al. 2003). Finally, these animals have

also demonstrated spatial memory deficit, hypoactivity and anxiety (Xue-Liang Li *et al.*, 2002). Other studies have also highlighted a role for CCK1R in memory. In healthy rats, injection of CCK-8 improves olfactory recognition and agonism of the CCK2R or antagonism of CCK1R impairs this. Whereas activating CCK1R positively modulates olfactory recognition memory (Lemaire *et al.*, 1992, 1994). In Japanese quail injecting CCK1R antagonist devazepide after the first memorisation session disrupted feed memory formation as quail perform significantly worse during the recall session after a 24h delay. Inhibiting CCK2R demonstrated no significant effect on recall although a similar trend was noted (Berthelot *et al.*, 1996). Finally, enterostatin, a pentapeptide released from the pancreas, was demonstrated to improve mouse memory on a passive avoidance test. This effect was inhibited by pre-treatment with the CCK1R antagonist lorglumide. Enterostatin did not show affinity for CCK1R itself indicating that its memory enhancing effects were caused by CCK production which in turn signalled through CCK1R (Ohinata *et al.*, 2007). Altogether this data shows that signalling through CCK1R has memory-related effects and blocking this receptor impacts many types of memory.

Upon examination of the CCK1R signalling pathway interesting theories may be posited about its abilities as an inducer of cell survival. CCK1R is known to signal via PI3K/Akt pathway (Zhou *et al.*, 2014) a known cell survival pathway (**Figure 7**). In fact in a study on Ewing tumour growth, inhibition of CCK1R with devazepide inhibited cell tumour growth and induced apoptosis of this cell type (Carrillo *et al.*, 2009). Further CCK has been linked to stimulating proliferation in CHO cells via the PI3K pathway (Buscail *et al.*, 1995). CCK is up-regulated in obesity and it was found that a loss of CCK in C57BL/6-Leptin(ob/ob) mice decreased beta-cell mass and pancreatic islet size through beta-cell death. In culture, CCK was able to rescue these cells from cytokine and endoplasmic reticulum stress-induced death. It is suggested this pro-survival mechanism is mediated via CCK1R (Lavine *et al.*, 2010).

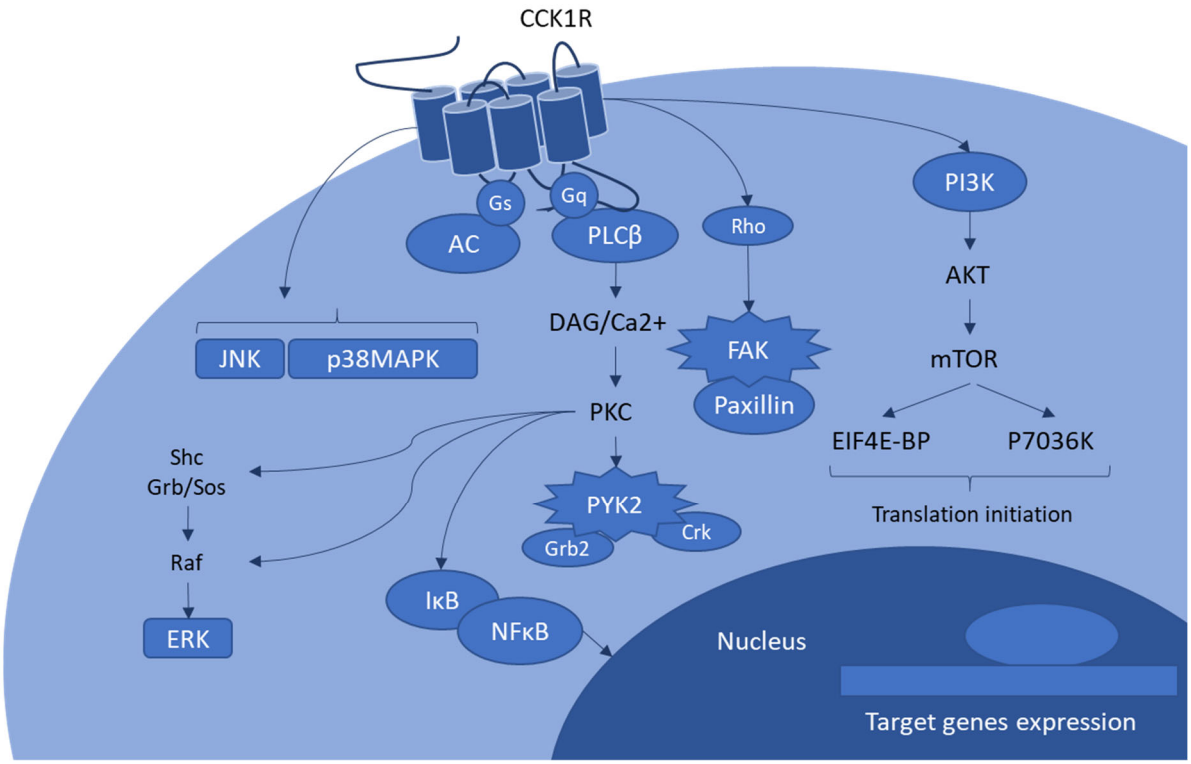

Figure 7. Downstream signalling pathways of CCK1R. CCK1R signals via the PI3K/Akt/mTOR pathway a known pro-survival pathway as well as Mitogen-activated protein kinases (MAPKs) such as ERK, JNK and p38MAPK which are linked to cell growth, differentiation and survival (Dufresne, Seva and Fourmy, 2006). Gs = G protein of the s family, Gq = G-protein of the s family, PLC = phospholipase C, DAG = diacylyglycerol, PKC = protein kinase C, PYK = protein tyrosine kinase, Crk = adapter molecule Crk, IκB = Initiator of κB, NFκB = nuclear factor κB, Src = tyrosine-protein kinase Src, Grb = growth factor receptor-bound protein, Sos = son of sevenless homolog, Raf = rapidly accelerated fibrosarcoma, ERK = extracellular signal-related kinase, FAK = focal adhesion kinase, PI3K = phosphatidylinositol 3-kinase, AKT = protein kinase B, mTOR = mammalian target of rapamycin, EIF4E-BP = eukaryotic translation initiation factor 4E binding protein, P70S6K = ribosomal protein S6 kinase, AC = adenyl cyclase, JNK = c-jun NH_2-terminal kinase, MAPK = mitogen-activated protein kinase.

Rats have been shown to have age-dependent responses to injections of CCK. Young and old rats demonstrate anorexia when injected with CCK in the periphery; however, middle-aged rats did not, hinting at a higher threshold for CCK, which may indicate an explanation for obesity in middle age in other animals. Induced obesity accelerated CCK-resistance in middle age and high sensitivity in old age, whereas calorie restriction prevented development of resistance in old age. Young rats given a CCK injection into the brain demonstrate an anorexic effect but this gradually disappears by the time they reach old age (Balaskó *et al.*, 2012, 2013). This gives an indication that CCK has a different role within different parts of the body and the brains control over CCKs satiety effects are not as significant in old age compared to peripheral components. However, earlier work suggests that injections of CCK or

CCK1R antagonist devazepide, had greater impact on glucose intake in older rats compared to younger ones. In old rats CCK inhibited glucose intake in a dose-dependent fashion, this effect was lessened in young rats and was not dose dependent. Devazepide however had no effect on young rats but increased glucose intake in old rats (Salorio *et al.*, 1994). This conflicting data shows that whilst there may be an age-related effect on CCK1R its modulation may vary in different systems. In humans either an increase in circulating CCK or sensitivity to CCK is blamed for age anorexia, the common phenomenon of reduced appetite and body weight during old age (Moss *et al.*, 2012). Which would suggest a change in response to CCK with age. However, a study on the effects of dexloxuglumide, a CCK1R antagonist, suggested no age or gender-related changes in response to treatment in humans (Roy *et al.*, 2005). As such changes in CCK in humans may be related to CCK2R changes or, as suggested by the data from rats, system specific variations. Though little work has been done on CCK in AD a recent study showed patients with higher CCK levels in CSF were seen to have decreased incidence of MCI or AD, better memory scores and higher grey-matter volume, specifically in posterior cingulate cortex, parahippocampal gyrus and medial prefrontal cortex (Plagman *et al.*, 2019).

Altogether this evidence suggests that CCK1R has a potential role in memory, cell health and alterations with age may lead to impaired signalling. Further a recent patient study suggests increased CCK may be beneficial for protecting against AD. As such I propose that agonism of the CCK1R could promote neuronal cell health and protection within AD and further may be beneficial for memory.

3.6 Conclusion

Whilst there are many theories of ageing with varying levels of evidence supporting them no individual idea has been able to encompass and explain the events of ageing in an overarching theory. This thesis suggests this is because the study of ageing has focussed on changes at a short-term cellular level and assumes this filters up to system change, whereas addressing ageing as a slow long-term change in system interactions through endocrines may produce a fuller picture of events. As such it is postulated that diseases of ageing, such as Alzheimer's disease, should be addressed from this

standpoint with a view that using these hormonal interactions may provide new avenues of treatment strategies which can produce the disease halting effects that previous treatments have failed to elicit. As such I will explore the potential of two hormone-based molecules for their neuroprotective and cognitive enhancing abilities to assess their validity as potential AD treatments.

4 METHODS

Unless otherwise stated all reagents were from Merck (UK) and consumables from VWR (UK).

4.1 CELL CULTURE

4.1.1 Maintenance

SHSY-5Y human neuroblastoma cells (ECACC, UK) or HT22 mouse hippocampal neuronal cell line (Merck, UK) were seeded in either a small cell culture flask (25cm^2), containing 5 ml of maintenance medium, or a large cell culture flask (75cm^2), containing 10ml of maintenance medium. Maintenance medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (4500µg/l), 0.1% (v/v) penicillin (10,000 units/ml) streptomycin (10mg/ml) (PenStrep) and 10% (v/v) iron fortified calf serum (FCS). Cells were kept in a HERAcell 150i incubator (ThermoFisher Scientific, UK) at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Once cells had reached 70% confluency all medium was removed from flasks, cells were washed using Hank's buffered salt solution (HBSS) and stripped using 429µM trypsin (Worthington, USA; SHSY-5Y cells) or 1x TryPLE (HT22 cells). Once cells were detached from the flask substrate, maintenance medium was added back to the flask to suspend the cells for removal. 10µl of cell containing medium was mixed with equal volume of trypan blue so that the number of viable cells could be assessed using a haemocytometer. Cells were then diluted and plated on sterile nunc coated plates at an appropriate density (see **Table 2** for SHSY-5Y seeding densities). HT22 cells proliferate at a much higher rate and it was found that these cells were always 100% confluent in flasks when being plated for experimentation. As such these cells were diluted 1:60 directly from the flask for use. SHSY-5Y cells were left to settle for 48h before use, HT22 cells were left for 24h.

Plate type	Cell density	Medium volume/well
Undifferentiated cells 96 well plate	$1x10^5$ cells/ml	100µl
Differentiated cells 96 well plate	$6x10^4$ cells/ml	100µl
48 well plate	$3x10^4$ cells/ml	400µl
24 well plate	$5x10^4$ cells/ml	500-700µl
35mm dishes	$3x10^5$ cells/ml	1.5ml
60mm dishes	$8x10^5$ cells/ml	5ml

Table 2. SHSY-5Y cell seeding densities and medium volume for different cell plates.

4.1.2 Differentiation

SHSY-5Y cells were differentiated to a neuronal phenotype for use in many of the experiments. 48h after plating maintenance medium was replaced with DMEM supplemented with 1% (v/v) FCS, glucose (4500µg/l), 0.1% (v/v) PenStrep (10,000units/ml and 10mg/ml) and 10µM retinoic acid and incubated for 5days to initiate differentiation to a neuronal phenotype with a 50% medium change after 48h. This medium was then changed to a serum replacement medium consisting of: DMEM, 2% serum replacement 2 (SR2, Sigma, UK), 80µM 5-fluorodeoxyuridine (5-fdu), glucose (4500µg/l) and 0.1% (v/v) PenStrep (10,000units/ml and 10mg/ml) and incubated for 7 days with a 50% and a 100% medium change after 48h and 96h respectively. Many methods of SHSY-5Y differentiation involve maintenance in low levels of serum and introduction of neurotrophic factors, however the maintenance of cultures in these factors when investigating hormonal based treatments is problematic as they can act via and modulate the same signalling pathways (Shipley, Mangold and Szpara, 2016; Teppola *et al.*, 2016; Song *et al.*, 2017). As such SR2 was chosen for long-term differentiated cell culture maintenance as it does not contain any growth factors or steroid hormones but provides the nutrients needed for cell survival under normal conditions. 5-fdu is a mitotic inhibitor which eliminated undifferentiated cells still entering the cell cycle. Cells were maintained in this medium for 1 week prior to use in experiments in order to negate the innate protective qualities of retinoic acid (Cheng *et al.*, 2013).

Differentiation of HT22 cells was induced 24h after plate down by changing medium to DMEM supplemented with glucose, PenStrep, 2% (v/v) SR2 and 100µM cyclic adenosine monophosphate for 24h (Inda *et al.*, 2017).

4.1.3 Ageing SHSY-5Y cell cultures

For ageing cell cultures SHSY-5Y cells were plated in the central 24 wells of 48 well plates at a density of $5x10^4$ cells/ml with 400µl of cell suspension added per well. The outer wells were filled with 1ml of PBS in order to counteract evaporation of medium from the central wells over their incubation period. All culture media were made in the same manner as for differentiating SHSY-5Y cells normally, however, PenStrep was replaced with 1% (v/v) Fungibact (penicillin-streptomycin-neomycin; 5000units, 5mg and 10mg per ml respectively) as the length of culturing these cells made them more vulnerable to contamination. For differentiating ageing cells, they were left in retinoic acid medium for 7 days rather than 5 with two 50% medium changes every 2-3 days as this led to a greater number of cells differentiating into a neuronal phenotype and hence better survival rate. These cells were left in mitotic inhibitor for one week prior to first treatment as described above (see **4.1.2**). At the point of first treatment the volume of medium per well was increased to 700µl as this helped protect cells from medium evaporation and created more of a cushion against the physical stress of medium changes with time. After the treatment was first added the cells 50% medium changes twice a week, however, to account for medium loss 300µl medium was removed and 400µl of fresh medium containing treatment was added. Outside wells containing PBS were topped up during medium changes at the experimenter's discretion.

4.2 INDUCING CELL DEATH

4.2.1 Heatshock

To cause a rapid stress response and necrosis within cell cultures, plates sealed with nescofilm were placed in a water bath and heated to 60°C for 2 minutes. Plates were then taken out, film removed

and allowed to cool in the cell culture hood for 5 minutes. After cooling treatments were added directly to the cell culture wells. These cultures were assayed after 24h.

4.2.2 Combined serum and glucose deprivation (SGD)

Depriving cells of serum and glucose invokes a starvation response and apoptosis. This is also used as an in vitro model of ischemia as it is thought to reflect the molecular mechanisms in neurons seen with ischemia-induced brain injury (Mousavi *et al.*, 2016). 48h after plate down all medium was removed from SHSY-5Y cells and cells in all conditions were washed 2-3 times with HBSS. Control cells had maintenance medium replenished whereas cells in combined serum and glucose deprivation (SGD) condition were given DMEM supplemented with only 0.1% (v/v) PenStrep (10,000units/ml and 10mg/ml). Cells were kept in these conditions for 24, 48 or 96h. For any treatment period longer than 48h cells a 50% medium change occurred halfway through the period.

4.2.3 Copper chloride

Abnormal levels of copper, such as in Parkinson's (Montes *et al.*, 2014), Alzheimer's (Brewer, 2015) and Wilson's disease (Stock *et al.*, 2016), have been shown to increase ROS and apoptosis (Megger *et al.*, 2017). For induction of cell death, copper chloride was used at a concentration of 10µM dissolved in mitotic inhibitor medium (Panjehpour, Taher and Bayesteh, 2010). SHSY-5Y cells were treated after differentiation with retinoic acid and maintenance in mitotic inhibitor medium for 1 week. Cultures were treated with copper chloride for 96h with 50% medium change after 48h.

4.2.4 Amyloid Beta$_{1\text{-}42}$

Amyloid $\beta_{1\text{-}42}$ (Aβ) has been identified as having a major role in AD and can cause neuronal death (Krishtal *et al.*, 2017). For the induction of cell death lyophilised Aβ powder was solubilised in dH_2O to a concentration of 1mM in PBS and incubated for 24h at 37°C. This generates the toxic β-sheet confirmation (Simmons *et al.*, 1994). Aβ was used at a working concentration of 10µM dissolved in mitotic inhibitor medium. SHSY-5Y cells were treated after differentiation with retinoic acid and

maintained in mitotic inhibitor medium for 1 week. Cultures were treated with Aβ for 96h with a 50% medium change after 48h. For the induction of stress response Aβ was used at a concentration of 1μM.

4.2.5 Protective Reagents

In order to assess the effectiveness of leptin fragment 116-130 ($leptin_{116-130}$) as a protective reagent it was initially compared against human leptin and mouse leptin. $Leptin_{116-130}$ is derived from murine leptin. To confirm that a human based fragment of similar amino acids would induce the same effects $leptin_{116-130}$ was later compared to $leptin_{117-125}$ and to smaller hexamers for leptin from the same region $leptin_{117-122}$ and $leptin_{116-121}$.

A71623 the CCK1R receptor agonist was assessed for its neuroprotective effects initially on undifferentiated SHSY-5Y and HT22 cells and then on differentiated SHSY-5Y cells.

4.3 Viability Assays

4.3.1 LDH

Lactate dehydrogenase (LDH) assays were carried out on cell culture medium after treatment period to ascertain membrane permeability. LDH is an enzyme which is usually found within cell cytosol, however during cell death by necrosis, or late in the apoptotic pathway, the membrane ruptures and, in culture, is released into the medium. Cell culture treatment media was removed from culture plates. Media from technical repeats was combined in a single Eppendorf and labelled appropriately. Aliquots were either frozen at -20°C for storage or assayed immediately. Prior to use media was centrifuged at 13000rpm for 10minutes in a Boeco M-24 centrifuge (Boeco, Germany) to remove any intact s-phase cells from suspension. Media was then pipetted in triplicate for each condition into a clean labelled 96 well plate (Sterelin, Fisher Scientific, UK). An equal volume of LDH reaction mix (0.13% (w/v) chemical mix (26% (w/w) Iodonitrotetrazolium chloride, 6.7% (w/w) phenozine methosulphate and 67% (w/w) β-nictotinamide adenine dinucleotide hydrate) dissolved in 0.2M Tris in dH_2O (pH8.2), 0.5% (v/v) lactic acid and 0.1% (v/v) Triton X-100 (Stockland and San Clemente, 1968)) was added to cell culture medium. Absorbance was read at 490nm on a Biohit BP100 plate reader (Biohit, Finland). Plates were

read once every 10 minutes until colour fully developed determined by waiting until maximum difference between cell death induced and untreated control conditions was reached or until readings in treatment conditions were higher than blanks. Readings normalized to control and averaged over experiments. Only plates where the kill condition had LDH readings more than 15% over control were deemed to have produced a sufficient effect for protection to be evident.

4.3.2 MTT

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a measure of mitochondrial activity within living cells. The MTT dye is taken up by cells and NADH reduces it to its insoluble formazan, this reaction will only occur in healthy viable mitochondria and hence gives an indication of cell viability (Barltrop *et al.*, 1991). A working concentration of 1mg/ml MTT was added to cell culture plates at the end of their treatment period. These cells were incubated for a further 1.5hours after which all medium was removed, and cells were solubilised with equal volume Dimethyl sulfoxide (DMSO). Once cells were completely dissolved Sorensen's glycine buffer (0.075% (w/v) glycine, 0.058% (w/v) sodium chloride, dissolved in dH_2O, pH 10.5) was added (1:5) to stabilise the reaction. Absorbance was read at 570nM on a Biohit BP100 plate reader. Data were normalised to control to adjust for differences in plating density and averaged over experiments. Only plates where to kill condition had MTT readings 15% lower than control were deemed to have produced sufficient effect for protection to be evident.

4.3.3 Crystal Violet

Crystal violet (CV) is a dye which will bind to ribose molecules such as DNA and nuclei, as well as proteins. This can be used as a measure of cell viability as dead cells will detach from the culture plate and hence will be removed with culture medium and washing steps, leaving stained viable cells adherent to the plates which can then be solubilised and read for absorbance (Feoktistova, Geserick and Leverkus, 2016). Cells were fixed in neutral buffered formalin (NBF, 10% (v/v) 37-40% formaldehyde solution diluted in phosphate buffered saline (PBS, 0.85% (w/v) sodium chloride, 0.04% (w/v)

dihydrogen sodium phosphate, 0.1% (w/v) disodium hydrogen phosphate, dissolved in dH_2O, pH 7.2)) for at least 15minutes, then washed 3 times for 5minutes in PBS prior to staining with crystal violet staining solution (2% (v/v) crystal violet solution, 3% (v/v) 100% ethanol, dissolved in dH_2O) for 30 minutes. Plates were washed in dH_2O until no more purple colour was released, then solubilised in equal volumes of 10% SDS (10% (w/v) Sodium dodecyl sulphate dissolved in dH_2O) before reading the absorbance at 570nM on a Biohit BP100 plate reader. Data were normalised to control and averaged over experiments. Only plates where the kill condition had CV readings 15% lower than control were deemed to have produced sufficient effect for protection to be evident.

In ageing SHSY-5Y cultures the assay was carried out in the same manner, however as cells were more fragile and hence susceptible to being dislodged by repeated washing, only one wash with PBS after fixing with NBF was used but for at least 30 minutes to try and remove as much excess fix as possible. After staining with crystal violet staining solution plates were only washed once with dH_2O and when this was removed wells were assessed for cell loss due to mechanical stress (the plates were turned upside down over white paper towel to remove dH_2O and if significant pieces of purple cell matter were seen on the tissue from a certain well this well was removed from analysis). 1-2 of the outer wells containing PBS were used as a blank for these crystal violet assays and were treated in the same way as the cell containing wells. This was done because the fragile nature of these cells prevented thorough washing of excess CV stain and these blank wells allowed this to be accounted for in data analysis.

4.4 NBT ASSAY

The nitroblue tetrazolium (NBT) assay can be used to measure the presence of superoxide in cell cultures. The NBT is reduced by superoxide forming blue formazan deposits which can be solubilised, and absorbance measured (Sim Choi *et al.*, 2006). At the end of the treatment period sterile filtered nitrotetrazolium blue chloride dissolved in PBS was added at 0.05% (v/v) into cell culture medium and incubated for 2 hours. All medium was removed, and cells were solubilized in equal volumes of DMSO

on a Heidolph rotamax 120 rocker, speed 3 (Heidolph, Germany) until a pale purple colour developed, up to a maximum of 10 minutes. 1M potassium hydroxide was added in equal volume to DMSO and absorbance of the blue product was read immediately at 570nm on a Biohit BP100 plate reader. Data were normalised to control and averaged over experiments.

4.5 Cell Staining

Cells for imaging were plated on borosilicate glass coverslips (Pyramid Innovation, UK). For phase contrast photomicroscopy cells were fixed in NBF for 15 minutes and washed 3x 5minutes in PBS. Images were then taken under phase contrast illumination on a Nikon eclipse TS100 microscope using a Visicam 3.0 camera.

4.5.1 DAF-2DA

4,5-Diaminofluorescein (DAF-2DA) is an indicator of nitric oxide (NO). When the DAF2-DA dye is taken up by the cell it is hydrolysed to DAF2 which reacts with NO to form a fluorescent triazolofluorescin with an excitation wavelength of 450-490nm (Kojima *et al.*, 1998; Kasim, Branton and Clarke, 2001). At the end of the treatment period culture medium was removed and replaced with fresh medium containing 1µM DAF2-DA (Insight, UK)(Strother, 2018) and incubated for 45minutes. Cultures were then fixed in NBF for 15minutes and washed 3x 5minutes in PBS. Coverslips were mounted on slides with mountant (1%(w/v) N-propyl-gallate dissolved in 80% (w/v) glycerol, 20% (w/v) PBS) and sealed with nail polish. Slides were imaged immediately on a Zeiss Axio MR2 microscope or frozen at -20°C for later analysis. Post-imaging intensity was measured using image J and the corrected total cell fluorescence (CTCF) of cells was calculated. CTCF = integrated density of selected cell – (area of cell x mean background) (Burgess *et al.*, 2010; McCloy *et al.*, 2014). To work out the mean background three random areas of a selected image not containing any cells were selected and their integrated density and area calculated. For each area the integrated density was divided by area and averaged over the three random areas.

4.5.2 DAPI

4',6-diamidino-2-phenylindole (DAPI) is a DNA stain which can be used both to detect cellular nuclei and elucidate mitotic or cell death status (Filippi-Chiela *et al.*, 2012). During mitosis chromosomes line up at the cell equator and chromatids are pulled apart into separating daughter cells, DAPI staining allows us to capture this and allows for classification. Additionally, during apoptosis the cell nucleus becomes condensed, pyknotic and undergo chromatolysis all of which can be seen with DAPI staining (Majno and Joris, 1995; Mandelkow *et al.*, 2017). After treatment culture medium was removed and cells were fixed in NBF for 15 minutes and washed 3x 5minutes in PBS. A working concentration of 0.5µg/ml DAPI was diluted in PBS and added to the cells for 15minutes. Cultures were then washed a further 3x 5 minutes in PBS. Coverslips were mounted (see 4.5.1) on slides and sealed with nail polish, then imaged on Zeiss Axio MR2 microscope or frozen at -20°C for later imaging and analysis. DAPI stained nuclei were judged for normal, apoptotic and mitotic morphology.

4.5.3 H_2DCFDA

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) is a live cell dye which fluoresces in the presence of ROS (Krejsa and Schieven, 2000). The diacetate group is cleaved by cellular esterases and oxidation converting the non-fluorescent H_2DCFDA to 2',7'dichlorofluorescein (DCF) which is highly fluorescent. Cells were stained with H_2DCFDA (Sigma, UK) for detection of oxidative stress were incubated with the dye at 10 µM in culture medium for 45minutes at the end of the treatment period and live imaged immediately on a Leica LS2 Confocal. Post-imaging intensity was measured using image J and the CTCF of cells was calculated.

4.5.4 Immunocytochemistry

Immunocytochemistry allows fluorescent identification of specific proteins within cell cultures using antibodies and fluorescent secondary antibodies (Maxwell and Salto-Tellez, 2016). After treatment all cell culture medium was removed, and cells were fixed in NBF for 15 minutes then washed 3x 5minutes in PBS. Non-specific antibody binding was then blocked with Horse serum block (HSB, 10%

horse serum in PBS containing 0.01% Triton X-100 (PBS-T)) before overnight incubation with the primary antibody (diluted in HSB at the appropriate concentration, Table 3) at 4°C. Cells were washed 3x 5 minutes in PBS then incubated for 1 hour at room temperature with a fluorescent secondary, diluted in HSB at the appropriate concentration (Table 3). Cells were washed again 3x 5minutes in PBS then mounted, before imaging with a Zeiss Axio MR2 microscope.

4.6 Protein Extraction

4.6.1 From cell culture

To extract the soluble protein fraction from cell cultures, after treatment period all culture medium was removed and 500µl of protein extraction buffer (Tris-buffered saline (TBS; made according to manufacturer's instructions) pH 7.5 containing 1% (v/v) SigmaFAST protease inhibitor cocktail (diluted according to manufacturer's instructions; to inhibit proteases released from cells during lysis which would degrade target proteins) and 1% (v/v) triton-X 100 (to lyse cells)) was added to each dish. A cell scraper was then used to dislodge cells from the substrate and suspend them in the extraction buffer, which was removed and placed into microfuge tubes. An additional microfuge tube containing only 500µl of extraction buffer was also made and labelled as an extraction control for each experiment. This was to control for any contamination of the extraction buffer. All microfuge tubes were then centrifuged in a Boeco M-24 (Boeco, Germany) at 13,000 rpm for 10 minutes. The supernatant was removed, aliquoted and frozen at -20°C for storage.

4.6.2 From fixed tissue

Tissues extracted by this method include hemispheres of mice after OPC testing from saline, leptin, leptin$_{116\text{-}130}$ and A71623 treated groups, as well as from healthy male Sprague dawley rats of ages: 14-19days (juvenile), 21-28 days (post-weaning), 42-60 days (adult) or 12-14 months (aged) donated by Dr J. Harvey, University of Dundee. Throughout this protocol all tissues and reagents were defrosted and kept on ice as much as possible. Tissue was first weighed to calculate the appropriate volume of lysis buffer necessary for each section. 1ml of final lysis buffer (92%(v/v) lysis buffer (48%

(w/v) urea (chaotropic agent, breaks hydrogen bonds), 4.5% (w/v) 3-[(3-Cholamisopropyl)dimethylammonio]-1-propanesulfonate (solubilise membrane proteins and receptors), 1% (w/v) dithiothreitol, 96.75% dH_2O (reducing agent prevents folding and formation of disulphide bonds)), 4% (v/v) protease inhibitor cocktail, 4% (v/v) dH_2O) was added per 0.25g of tissue. Tissue was then homogenised and triturated with a 1ml pipette and needle (BD Microlance 3 25g 5/8 inch) and syringe (NORM-JECT; Henke-Sass Wolf, Germany) allowing the tissue to rest in final lysis buffer on ice periodically to assist in tissue breakdown. Tissue was left for a further 15minutes once judged to be suitably homogenised and re-triturated with a syringe to ensure maximum protein extraction. Samples were centrifuged in a Boeco M-24 centrifuge at 12,000 rpm for 5 minutes and supernatant removed for use. The supernatant was vortexed (Yellow line TTS2; IKA-Werke, Germany) for 20seconds per tube at 2.5 rpm, aliquoted and stored at -20°C.

4.7 BRADFORD ASSAY

The Bradford assay was used to determine protein concentration. This assay is based on the Coomassie blue dye which is brown in the absence of protein but once bound is converted to a blue product (Sapan, Lundblad and Price, 1999). The absorbance of this blue product can be detected and analysed. A greater absorbance in this assay indicates a greater protein concentration. A standard curve was prepared using 1:10 serial dilutions of 10mg/ml bovine serum albumin to give a range from 1ng/ml-10mg/ml. To a 10µl sample of each concentration of the standard curve and to 10µl of each protein sample 10µl 1M sodium hydroxide (to solubilise the protein) was added. 500µl Bradford reagent (1% w/v Coomassie blue, 5% w/v 95% alcohol, 10% w/v 85% phosphoric acid, in dH_2O) was added to each sample, mixed and loaded into 96 well plate (Steralin, Fisher Scientific, UK) and read at 590nm on a Biohit BP100 plate reader. The standard curve was plotted on a logarithmic scale in excel and the straight-line equation (y=mx+c) was reverse engineered to work out the protein concentration of each sample (x) and the result anti-logged. The R^2 value was assessed for each line of best fit and only data

where at least four of the data points for the standard curve gave a line of best fit with a value of 0.9 or greater were used.

4.8 ELISA

The enzyme-linked immunosorbent assay (ELISA) was used to quantify proteins present in extracted samples using antibodies. Protein samples were denatured in boiling water for 10 minutes before being diluted to 7µg/ml (Thermo Fisher Scientific Inc., 2011) in ELISA coating buffer (BioLegend, USA) 20µl of sample was then loaded in triplicate (2 wells for protein of interest, 1 well for protein loading control) onto 96 well MaxiSorp Nunc-Immuno plates which were covered and incubated overnight at 4°C. Once coated all liquid was removed from the wells and replaced with 5% milk block (5% (w/v) dried milk powder dissolved in TBS with 1% (v/v) triton-X (TBS-T)), to block non-specific antibody binding, for 1 hour on a rocker. Primary antibodies diluted in 5% milk block (Table 3) were then added and plates incubated overnight at 4°C. Wells were then washed in 3x10minutes changes of TBS and secondary antibody (Table 3) added, diluted in 5% milk block for 1 hour at room temperature on a Heidolph rotamax 120 rocker, speed 3 (Heidolph, Germany). Wells were washed again in 3x 10minute changes of TBS and finally ABTS (KPL, USA) or TMB (Abcam, UK) substrate detection reagent were added to each well and plates covered with tin foil until a blue colour develops. Secondary antibodies are labelled with horseradish peroxidase (HRP) which reacts with these substrates, producing a colorimetric change for detection of secondary binding and hence indirectly measure protein levels. The reaction was stopped with 0.1M (ABTS) or 1M (TMB) hydrochloric acid and the plate was read at 630nm (ABTS – blue colour remains) or 490nm (TMB – yellow product forms) on a Biohit BP100 plate reader. Data was normalised to a loading control to adjust for variation in protein loading and assessed in comparison to a primary antibody lacking control to ensure results were due to primary and secondary antibody binding.

To measure the leptin levels in mouse brains following episodic memory testing a Mouse Leptin ELISA kit (Sigma, UK) was used. Following protein extraction concentrated whole mouse brain was

added to the provided plates and assayed in accordance with manufacturer's instructions. Each sample was run in duplicate. Similarly, commercially available kits were used to determine ratios of pan-STAT3 to phospho-STAT3 (Sigma, UK) and pan-Akt to phospho-Akt (Sigma, UK) from cell cultures treated with leptin and leptin fragment. Data were compared to a standard curve run alongside samples and final protein concentrations deduced.

4.9 Western Blotting

Western blotting was used to analyse proteins present by separating out molecular weight bands and using antibodies and chemiluminescence to visualise proteins more specifically. Protein samples were diluted in 6x sample buffer (375mM Tris-HCl pH6.8, 6% (w/v) SDS, 4.8% (v/v) Glycerol, 9% (v/v) 2(β)-mercaptoethanol, 0.03% (w/v) bromophenol blue, dissolved in dH_2O). Using the Lonza PAGEr Minigel Chamber (Lonza Group, Switzerland) system 12µl of each protein sample and 5µl of protein ladder (PAGE-MASTER protein standard (for SDS-PAGE); GenScript, USA) were loaded into wells of 2-4% polyacrymide gels (NuSep, Generon, UK) and run using cold running buffer (Tris-Glycine buffer (25mM Tris, 192mM glycine, pH 8.3)) using a Bio-Rad PowerPac 200 (Bio-Rad, USA) at 200V for 50minutes. Sponge, gel, chromatography paper and nitrocellulose membrane were then soaked in transfer buffer (Tris-Glycine-SDS buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS, pH 8.6) + 20% (v/v) methanol) for 10 minutes. Transfer cassettes were constructed as per manufacturer's instructions and run at 100V for 90 minutes in 1 litre of cold transfer buffer. Membranes were removed from cassettes and soaked in ponceau S (0.11% (w/v) ponceau s, 0.1% (v/v) acetic acid, dissolved in dH_2O) until protein bands appeared to confirm protein transfer. Membranes were labelled in pencil at this point to indicate location of ladder and top of membrane. Ponceau S was removed and filtered for reuse. The membrane was washed in three quick changes of TBS followed by 3 x 5 min washes until ponceau s staining was removed. 5% milk block solution was added and membrane was incubated for 15 minutes on a Heidolph rotamax 120 rocker, speed 3 (Heidolph, Germany) in order to prevent non-specific binding, after which the primary antibody was added at appropriate concentration (**Table 3**), diluted in 5% milk block

solution and left to incubate at 4°C overnight or 2hours at room temperature on the rocker. Following this, the membrane was washed 3x 5 minutes in TBS and secondary antibody was added at the appropriate concentration (Table 3), diluted in 5% milk block and left on the rocker for 1hour. Finally, the membrane was washed a further 3x 5min in TBS and bands were detected using ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology, USA). The membrane was imaged using the ChemiDoc-it^2 (UVP) system and intensity quantified using imageJ. After imaging membranes were stripped by soaking in 10% acetic acid for 4x 15minutes and washed in multiple changes of TBS for 1 hour before re-probing. Membranes were probed a maximum of 3 times.

4.10 Fluorescent Immunohistochemistry

Immunohistochemistry allows fluorescent identification of specific proteins within tissue samples using specific primary antibodies and fluorescent secondaries. Samples used for this technique were cerebellum, parietal, occipital and rostral sections from 5 adult (4-9 years), 5 old (12-17 years) and 5 CDS (11-20 years old) cats, donated by Professor D. Gunn-Moore, University of Edinburgh. Tissue sections mounted on poly-lysine coated glass slides and labelled with pencil and fitted into metal histology slide racks, were then transferred through a series of solutions to clear and rehydrate the tissue as follows: histoclear I for 5 minutes, histoclear II for 5 minutes, 100% alcohol I for 3 minutes, 100% alcohol II for 3 minutes, 96% alcohol for 3 minutes, 70% alcohol for 3 minutes. Slides were washed in PBS for 5 minutes before being immersed in a plastic container of citrate buffer (0.1M citric acid pH 6.0). The container was sealed with cling film and placed in steamer for 25 minutes, for antigen retrieval. Slides cooled at room temperature until they could be handled, then placed in room temperature PBS to aid further cooling. Slides were washed in 2 changes of PBS for 5 minutes each then fitted into a sequenza rack (ThermoFisher, USA). 200µl 10% horse serum block (HSB, 10% heat inactivated horse serum in 90% TBS-T) was added to each slide and left for 20 minutes to block non-specific binding. 200µl of primary antibody diluted appropriately (Table 3) in 10% HSB was added to every slide but one (to act as the primary antibody lacking control, this slide had more HSB added at this step). Slides were

left to incubate overnight at room temperature. Next slides were washed in PBS for 3x 5minutes then 200µl of secondary antibody diluted appropriately (Table 3) in HSB was added to each slide (including the primary antibody lacking control) and incubated for 1hour in the dark to prevent bleaching of the fluorescent secondary antibody. Slides were washed in PBS for 3x5minutes then removed from their holders and mounted (see 4.5.1) by placing a 24x60mm glass coverslip over the top and sealed with nail polish. Slides were imaged immediately after nail polish had dried with a Zeiss Axio MR2 microscope. In addition to calculating CTCF, particle analysis was carried out on images taken from cat brains in image J. Particle analysis allows for quick detection and measurement of non-overlapping objects within an image, for example average size of cells within a field of view. For analysis of granule cells in the cerebellum stained for either ObR or CCK1R the granule layer was selected free-hand using the polygon selection tool. The following macro was applied to images individually using language IJ1Macro:

```
1 setMinAndMax(0, 600);
2 setAutoThreshold("Default dark");
3 //run("Threshold...");
4 setThreshold(280, 65535);
5 run("Analyze Particles...","size=0-0.5 show=Nothing display include summarize");
```

This code sets the black and white balance (1), thresholds the image to a user defined limit (2, 3, 4), as decided by the experimenter using three test images to decide the point at which the threshold excluded background noise and included positively stained cells, and finally analyses the area covered in positive staining (5). In order to exclude large particles which were part of blood vessels or neuronal processes from the Purkinje layer an upper size limit was used. For the ObR stained images the threshold was set at 18 – 65535 and for CCK1R stained images the threshold was set at 280-65535. For batch analysis of images from parietal, occipital and rostral sections, where the complete image was analysed the following code was applied to all open images:

```
1 #@ File(label = "Output directory", style = "directory") output
2 #@ String(label = "Title contains") pattern
3 processOpenImages();
4 function processOpenImages() {
5 	n = nImages;
6 	setBatchMode(true);
7 	for (i=1; i<=n; i++) {
8 		selectImage(i);
9 		imageTitle = getTitle();
10 		imageId = getImageID();
11 		if (matches(imageTitle, "(.*)"+pattern+"(.*)"))
12 			processImage(imageTitle, imageId, output);
13 	}
14 	setBatchMode(false);
15 }
16 function processImage(imageTitle, imageId, output) {
17 	setAutoThreshold("Default dark");
18 //run("Threshold...");
19 setThreshold(280, 65535);
20 run("Analyze Particles...", "include summarize");
21 }
```

This allows analysis of all open images (1-3) which match a user defined title parameter (4-16). A threshold was applied to images as described (17-19) and analysis of total area covered by positive staining was carried out (20-21).

4.11 ANTIBODIES

A range of antibodies were used throughout this thesis in a range of techniques. Here a table of the antibodies used, their manufacturers and the concentrations used in each technique (where appropriate) has been included (Table 3).

Antibody	ELISA	Western Blot	Immuno-cytochemistry	Immuno-histochemistry
NOS1 rabbit polyclonal (Santa Cruz)	1:1000	n/a	n/a	n/a
NOS2 rabbit polyclonal (Santa Cruz)	1:2000	n/a	n/a	n/a
NOS3 rabbit polyclonal (Santa Cruz)	1:500	n/a	n/a	n/a
Ob-R mouse monoclonal (Santa Cruz)	1:2000	n/a	1:1000	n/a
Presenilin 1 Rabbit (GenScript)	1:1000	n/a	n/a	n/a
Amyloid precursor protein Rabbit (GenScript)	1:2000	n/a	n/a	n/a
Endophilin 1 mouse monoclonal (Santa Cruz)	1:2000	n/a	n/a	n/a
Tau Rabbit polyclonal (Santa Cruz)	1:000	n/a	n/a	n/a
A-Tubulin mouse monoclonal (Santa Cruz)	1:2000	n/a	n/a	n/a
Phosphorylated Tau (Ser^{262}) Rabbit (GenScript)	1:5000	n/a	n/a	n/a
CCKAR Goat (Abcam)	1:3000	n/a	n/a	1:100
CCKAR Rabbit (Santa Cruz)	1:5000 (rat brain) 1:100,000 (cells)	1:250	1:1000	n/a

Leptin Receptor Rabbit (Abcam)	1:10,000	n/a	n/a	1:100
Anti-ERAB Mouse monoclonal (Abcam)	1:5000	n/a	n/a	n/a
Anti-ERAB Rabbit polyclonal (Sigma)	1:5000	n/a	n/a	n/a
Human/mouse Phospho-Akt mouse monoclonal (R&D systems)	1:1000	n/a	n/a	n/a
p-STAT3 goat monoclonal (Sigma)	1:1000	n/a	n/a	n/a
Mouse secondary	HRP conjugated (Sigma) 1:10,0000	HRP conjugated (Sigma) 1:10,000	FITC or Texas Red (Vector) 1:200	FITC or Texas Red (Vector) 1:200
Rabbit secondary	HRP conjugated (Sigma) 1:10,0000	HRP conjugated (Sigma) 1:5,000	FITC or Texas Red (Vector) 1:200	FITC or Texas Red (Vector) 1:200
Goat secondary	HRP conjugated (Sigma) 1:5,0000	HRP conjugated (Sigma) 1:5,000	FITC or Texas Red (Vector) 1:200	Dylight 488 IgG (Vector) 1:200

Table 3. Primary and secondary antibodies and their concentrations as used throughout this thesis

4.12 EPISODIC MEMORY TESTING

Episodic memory testing was carried out to elucidate any cognitive-enhancing effects of our compounds, leptin and leptin$_{116\text{-}130}$ in chapter 5 and A71623 in chapter 6. Male C57/BL6 mice (bred in house) were housed in groups of 2-5 on a 12h light/dark cycle with access to food and water *ad libitum*. Behavioural testing was carried out 4 days a week during the light phase under the project licence of Dr James Ainge (PPL7018306) and complied with national and international legislation for testing and maintenance of laboratory animals (Animals [Scientific Procedures] Act, 1986; European Communities Council Direction of 24 November 1986 [86/609/EEC]).

Testing was carried out in a 33 x 26 x 22.5cm box with removable wall and floor inserts to allow configuration into two contexts: plain and striped. The plain context had single colour painted walls and floor (white in the experiment testing leptin and leptin$_{116-130}$ (see 5.6) and dark grey in the experiment testing A71623 (see 6.11)) and the striped context had black and white vertical stripes with metal mesh overlaid on the floor. The box was in a fume hood in for the testing of leptin and leptin$_{116-130}$ but on a table in an open, darkened room in the testing of A71623 as part of method refinement. Extra-maze cues were consistent irrespective of the contextual configuration of the box but were more prominent in the A71623 experiment. Objects used were easily cleanable 3D household objects and were approximately the same size as a mouse in at least one dimension. These were fixed to the floor of the box using 3M Dual Lock reclosable fastener (3M, UK). Relative to the mouse's starting position objects were placed in the far left and right corners of the box with enough room surrounding each object for a mouse to comfortably circle the object. Mice were handled by the experimenter at least once daily for extended periods for 7 days prior to habituation. Habituation to the experimental context included handling animals in the hood or room used for testing (3 days), placing animals in a holding box which would be used between test conditions for 3 minutes individually (3 days), placing animals in the box used for testing for 3 minutes each with no objects (3 days per context), placing animals in the box used for testing for 3 minutes each with objects (3 days per context), colouring small section of tails with marker so as to identify individuals during experimentation (every other day). Testing then commenced in the following order: Novel Object Recognition (NOR), Object Place (OP), Object Context (OC) and Object Place Context (OPC) recognition tasks. Each task was tested over two days during testing of leptin and leptin$_{116-130}$ (see 5.6.1) and four days during testing of A71623 (see 6.11.1) and were counterbalanced for context, place and object across days, mice and treatment. Mice were only treated with compounds during the OPC task but NOR, OP and OC tasks were performed to ensure all mice were able to complete the individual elements of the OPC task. Testing was recorded via over-head camera and recorded on a laptop for later scoring and analysis. All sample and test phases were recorded. Each phase of each task lasted for 3 minutes with each mouse being tested for a maximum

of 15 minutes in a day. The box and every object were cleaned with F10 disinfectant (F10 products, UK) in between phases and animals in order to remove olfactory cues. New objects were used for each testing day and every task. A total of 26 and 38 objects were used in the testing of leptin and A71623 in these behavioural tasks, respectively.

4.12.1 Novel Object Recognition task

In the NOR task the sample and test phases took place in the same context. Context was counterbalanced across mice, days and object. In the sample phase a mouse was first placed in the box with two copies of an object, one positioned on the left and one on the right and allowed to freely explore for 3 minutes. After 3 minutes the mouse was removed by the experimenter and placed in the holding cage. The box and objects were wiped down with F10 and dried with tissue. One copy of the original object and one copy of a never before seen object were placed in the box, the mouse was returned and allowed to explore for 3 minutes (Figure 8). The mouse was removed from the box and returned to its home cage. The box and objects were wiped down and set up for the next mouse.

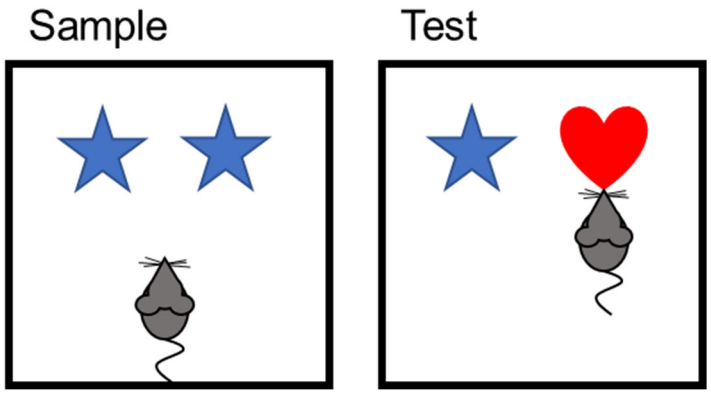

Figure 8. Novel Object Recognition task. A mouse was first placed in the testing box with duplicates of an object (blue stars) and allowed to explore for 3 minutes. The mouse was then removed and when returned to the box one of the objects had been changed for a new object (red heart). If the mouse recognised this as novel, we expected them to explore the new object for longer.

4.12.2 Object Place task

In the OP task the sample and test phases also occurred in the same context, which was counterbalanced across objects, day and mice. In the sample phase a mouse was placed in the box with two different objects and allowed to explore for 3 minutes. The mouse was removed, the box and

objects cleaned and two copies of one of the objects were placed in the box. The mouse was returned and allowed to explore for 3 minutes before being returned to its home cage (Figure 9). The box and objects were wiped down and set up for the next mouse.

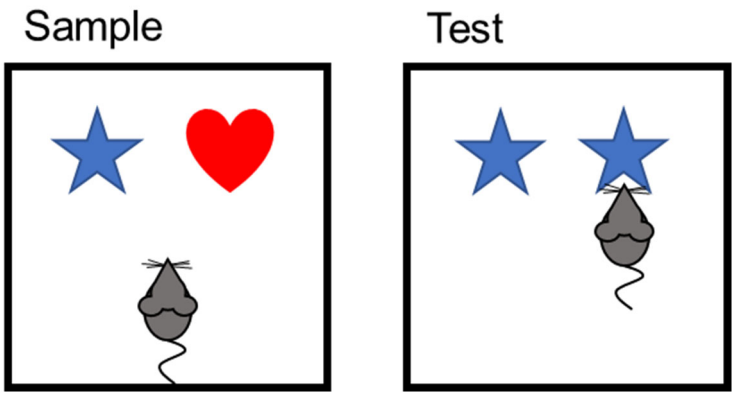

Figure 9. Object Place task. A mouse was first placed in the testing box with two different objects (blue star and red heart) and allowed to explore for 3 minutes. The mouse was removed and when returned one of the original objects appeared in duplicate (blue stars). If the mouse recognised that the object, shown here on the right, had never been in this place before, we expected it to explore this object for longer.

4.12.3 Object Context task

The OC task consisted of 2 sample and 1 test phase. The two sample phases each took place in a different context (plain or striped) and the test phase took place in one of the two contexts. The sample phases were counterbalanced for order of context and object shown across day and mouse. The test phase was counterbalanced for context, object and place of novel object across day and mouse. The mouse was first placed in the box set up in either context and with 2 copies of one object and allowed to explore for 3 minutes. The mouse was removed, the box and objects were wiped down and the context of the box was changed. 2 copies of a different object were then placed in the box for the second sample phase and the mouse was returned to the box and allowed to explore for 3 minutes. The mouse was placed in the holding cage, the box and objects were wiped down and one copy of each object seen were placed in the box in either context, depending on counterbalancing. The mouse was returned for the test phase and allowed to explore for a final 3 minutes before being returned to their home cage (Figure 10). The box and objects were wiped down and set up for the next mouse.

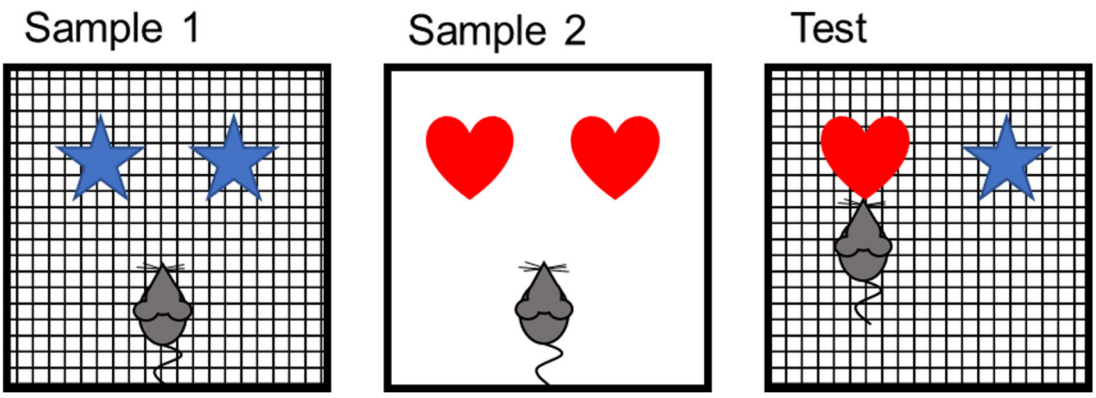

Figure 10. Object Context Task. A mouse was first placed in the testing box in context one with duplicates of an object (blue stars) and allowed to explore for 3 minutes. The mouse was removed and when returned to the box it was now in context 2 with duplicates of a different object (red hearts) and allowed to explore for 3 minutes. The mouse was removed and returned to the box a final time where one of each object was placed in one of the contexts. If the mouse recognised that it had never seen one of these objects in this context before, here the red heart in the striped context, then we expected it to explore this object for longer.

4.12.4 Object Place Context task

For the OPC task mice were injected intraperitoneally with treatment 30 minutes before their testing time, this allowed time for the drug to act and allowed the mice to recover from the procedure. In the experiments testing leptin and leptin$_{116\text{-}130}$ mice were injected by Dr J. Ainge. In the experiments testing A71623 injections were carried out by myself. The OPC task also consisted of two sample phases which each took place in a different context and a test phase which occurred in one of the two contexts. The sample phases were counterbalanced for context order, object order and place of object order. The test phase was counterbalanced for context, object, place and novel object, place and context order. The mouse was first placed in one of the two contexts with two different objects and allowed to explore for 3 minutes. The mouse was placed in the holding cage, the box and objects were wiped down, the context was changed and the place of the two objects was swapped. The mouse was returned to the box and allowed to explore for 3 minutes before being returned to the holding cage. The box and objects were cleaned again and two copies of one of the original objects were placed in one of the two contexts. The mouse was allowed to explore the box for a final 3 minutes before being returned to their home cage (**Figure 11**). The box and objects were wiped down and set up for the next mouse.

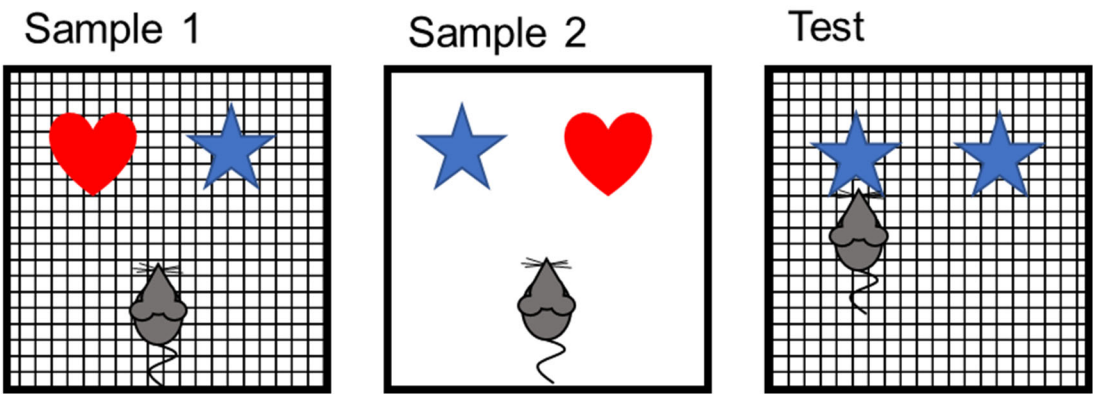

Figure 11. Object Place Context task. A mouse was placed into the testing box in sample phase 1 in context 1 with two different objects (a red heart on the left and a blue star on the right) and was allowed to explore for 3 minutes. The mouse was removed, the context was changed to context 2 and the objects switched position (red heart right and blue star left) and allowed to explore for 3 minutes. In the test phase the mouse was returned to the box set in either context (here context 1) and 2 copies of one object were placed in the box (blue stars). The mouse had seen this object before and seen it in both positions, however it had never seen the blue star on the left in context 1 before. If the mouse recognised this as a novel combination it would have explored the blue star on the left for longer than the blue star on the right.

For these data the time spent exploring novel and familiar object were recorded and total exploration time calculated. From this the discrimination index (d.i.) was calculated (d.i. = (novel object exploration time – familiar object exploration time)/total exploration time). Any experiments where mice explored for a total time of less than 5 seconds were discounted from the study.

4.13 Statistics

All statistical analysis was performed in GraphPad Prism 5. Data were tested for normality using Shapiro-Wilk tests. One-way analysis of variance (ANOVA) or Kruskal-Wallis tests for abnormally distributed data, with Dunnett's or Dunn's multiple comparison post-hoc tests; independent samples t-teats or Mann Whitney U tests for abnormally distributed data; one sample t-tests; or linear regression were used to determine differences between conditions where appropriate. Error bars represent the standard error of the mean. Statistical significance is denoted by asterisks as such: * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

5 A BIOACTIVE FRAGMENT OF THE LEPTIN HORMONE MIRRORS THE NEUROPROTECTIVE ACTIONS OF FULL-LENGTH HUMAN LEPTIN

5.1 INTRODUCTION

As discussed leptin has shown merits as a potential therapeutic in the realms of AD but there persist issues with the full-length molecule (see **3.4**) due to widespread receptor expression, for example in fat tissue (Kutoh *et al.*, 1998; Bornstein *et al.*, 2000), digestive system (Breidert *et al.*, 1999), vasculature (Trovati *et al.*, 2014; Lanier *et al.*, 2016), brain (Couce *et al.*, 1997; Bartolome Burguera *et al.*, 2000; van Swieten *et al.*, 2014) and glands (Glasow *et al.*, 1999; Jin *et al.*, 1999), treatment will generate off-target effects. Two of the most detrimental side effects are increased sensitivity to pain (Alvarez *et al.*, 2014; Younger *et al.*, 2016) and breast cancer (García-Robles, Segura-Ortega and Fafutis-Morris, 2013; Chang *et al.*, 2015; Rodrigo *et al.*, 2017). Additionally FDA-approved leptin recombinant Metreleptin, has received a black box warning for its risk of causing lymphoma and leptin antibodies (FDA, 2014; Diker-Cohen *et al.*, 2015).

In addition to side effects there are other drawbacks around the administration of the leptin hormone. Full-length leptin is a 16kDa peptide which in comparison to most drugs (e.g. aspirin at 180Da) is very large. Drugs that can be administered orally are usually small molecules with a molecular weight cut-off of around 500Da in accordance with the rule of five. The rule of five is a rough guide for evaluating a compounds likelihood to make an orally active drug. It states that such a drug should not violate more than one of the following criteria: no more than 5 hydrogen bond donors; no more than 10 hydrogen bond acceptors; a molecular mass less than 500Da; and a partition coefficient log *P* no greater than 5 (Lipinski *et al.*, 2001; Lipinski, 2004). These rules were largely developed from observations of solubility and permeability as factors in drug absorption. More recently successful orally active drugs have been produced which violate the rule of five, these are largely found in natural product metabolites (i.e. chemical synthesis of compounds produced by living organisms), however it is suggested these compounds have unique 'chameleon-like' abilities to change shape and polarity

depending on environment (Lipinski, 2016). Whilst there is some discussion on peptide drugs having exceptions to the rule of five even the largest of these only reach 43 amino acids in length (Santos, Ganesan and Emery, 2016), which is significantly smaller than the 167 of leptin, further native peptide hormones are susceptible to metabolism and elimination (Fotherby, 1996). As such leptin is difficult and costly to manufacture as well as forcing its administration to be via subcutaneous injection. Leptin and its recombinant analogue Metreleptin, are licensed for the treatment of obesity related to congenital leptin deficiency and for congenital or acquired (autoimmune) lipodystrophy, respectively (Paz-Filho, Mastronardi and Licinio, 2015; Friedman, 2016; Meehan *et al.*, 2016). Whilst leptin itself has shown some promise as an anti-obesity treatment its effectiveness between patients is variable (Heymsfield *et al.*, 1999) and as a monotherapy it largely failed due to leptin-resistance in obesity, as such leptin modifications have been explored to target the areas of resistance (Yi *et al.*, 2014). Metreleptin is a recombinant human leptin analogue of 147 amino acids used in the treatment of lipodystrophies. These conditions are characterised by a loss of adipose tissue and hence a decrease in leptin. Treatment with metreleptin was shown to be effective in counteracting many of the problems faced with lipodystrophy including dyslipidemia, insulin resistance and diabetes (Meehan *et al.*, 2016). However even these modified versions of leptin still have the caveat of daily injections which further adds to the cost of this treatment as a healthcare professional is required to carry out administration. Whilst self-administration may be possible this requires training, adding further to the costs (Mohr, Cox and Merluzzi, 2005) and a consideration for the target population of this treatment must be made as age-related loss of dexterity will impact their ability to self-inject (Keininger and Coteur, 2011). Further, subcutaneous injection can cause its own side effects including pain, inflammation and oedema (Fonzo-Christe *et al.*, 2005) and patients prescribed insulin assessed on their self-injection experience report them as a burden, as having a negative impact on their quality of life and 20% said they would skip injections they should take (Rubin *et al.*, 2009). Given that a leptin treatment for AD would likely be a disease halting measure rather than cure any patient prescribed it would be required to administer daily for the remainder of their life. As such a treatment would need to have high retention rate. In

general, oral medications have been reported as more convenient, having less side effects and are generally more preferable than injections (Gruffydd-Jones, Hood and Price, 1997; Eagle *et al.*, 2017; Holko, Kawalec and Mossakowska, 2018) and have been shown to have greater adherence than injectable treatments (Johnson *et al.*, 2017).

Therefore, for leptin to become a feasible AD treatment it would need to have disease modifying effects, be specific in its target action, be small enough to be manufactured as an oral drug and have potential for further modification to overcome premature metabolism. As such a fragment of the leptin protein could be used that emulates leptins neuroprotective effects without the negative widespread actions of the whole protein. Small naturally occurring and synthetic peptides are being explored for their uses both as means of delivering therapeutics (Ramsey and Flynn, 2015) and for their roles as therapeutics themselves (Marya *et al.*, 2018). This approach leads the way for previously promising treatments to be refined to best benefit the patient. A bioactive fragment of leptin ($leptin_{116\text{-}130}$) has already been discovered which has the potential to fulfil the criteria (Tena-Sempere *et al.*, 2000). This site was shown to be essential for leptins weight reducing effects (Grasso *et al.*, 1997; Rozhavskaya-Arena *et al.*, 2000) and mutations in this region of the leptin molecule led to an inability to activate the CRD of ObR (Peelman *et al.*, 2004) (see **3.4**).

This chapter aims to test the ability of a leptin fragment of amino acids 116-130 ($leptin_{116\text{-}130}$) to protect against neuronal death in response to neurodegenerative and AD-relevant conditions and compare it to the full-length leptin hormone. Further, as leptin has previously been shown to regulate biomarkers related to AD (Doherty *et al.*, 2013; Niedowicz *et al.*, 2013; Platt *et al.*, 2016), the abilities of $leptin_{116\text{-}130}$ to modulate these will be assessed. Experiments will be carried out *in vitro* using fully differentiated SHSY-5Y neurons as this model allows for easy biochemical manipulation within a human cell population with a clear neuronal morphology and the ability to generate action potentials and therefore form neural networks. Following this leptin and $leptin_{116\text{-}130}$ shall be investigated for potential memory enhancing effects in mice and therefore protective abilities in the murine hippocampal cell

line HT22. Finally, an assessment of hexamers from within the leptin$_{116-130}$ fragment shall undergo initial assessment for potential effects as molecules of this size allow for easier modification into therapeutics than even leptin$_{116-130}$.

5.2 THE LEPTIN RECEPTOR, OBR, IS PRESENT IN SHSY-5Y CELLS AND ITS EXPRESSION DOES NOT CHANGE WITH DIFFERENTIATION

To ascertain the presence of the leptin receptor, ObR, in the SHSY-5Y cell line and to observe if this changes with differentiation, untreated cultures were used at 48h post plate down (undifferentiated), after 5 days in retinoic acid (differentiated) and at 1 week post-differentiation (7 days in mitotic inhibitor medium) and were used for ELISA assay (Figure 12A) and immunocytochemistry (ICC; Figure 12B). This experiment was performed to ensure the receptor needed for leptin to enact its effects was present in the cells and that expression was still evident at the time points used for further experiments. For the ELISA assay protein was extracted from an n of 8 experimental repeats and Bradford assays performed to determine equal loading. ObR and α-tubulin primary antibodies with anti-mouse secondaries were used for the ELISA and ObR with anti-mouse Texas red secondary were used for the ICC as stated (See **Table 3** for antibody concentrations). Greater red fluorescence in the ICC indicates a greater detection of ObR, these images were visually compared.

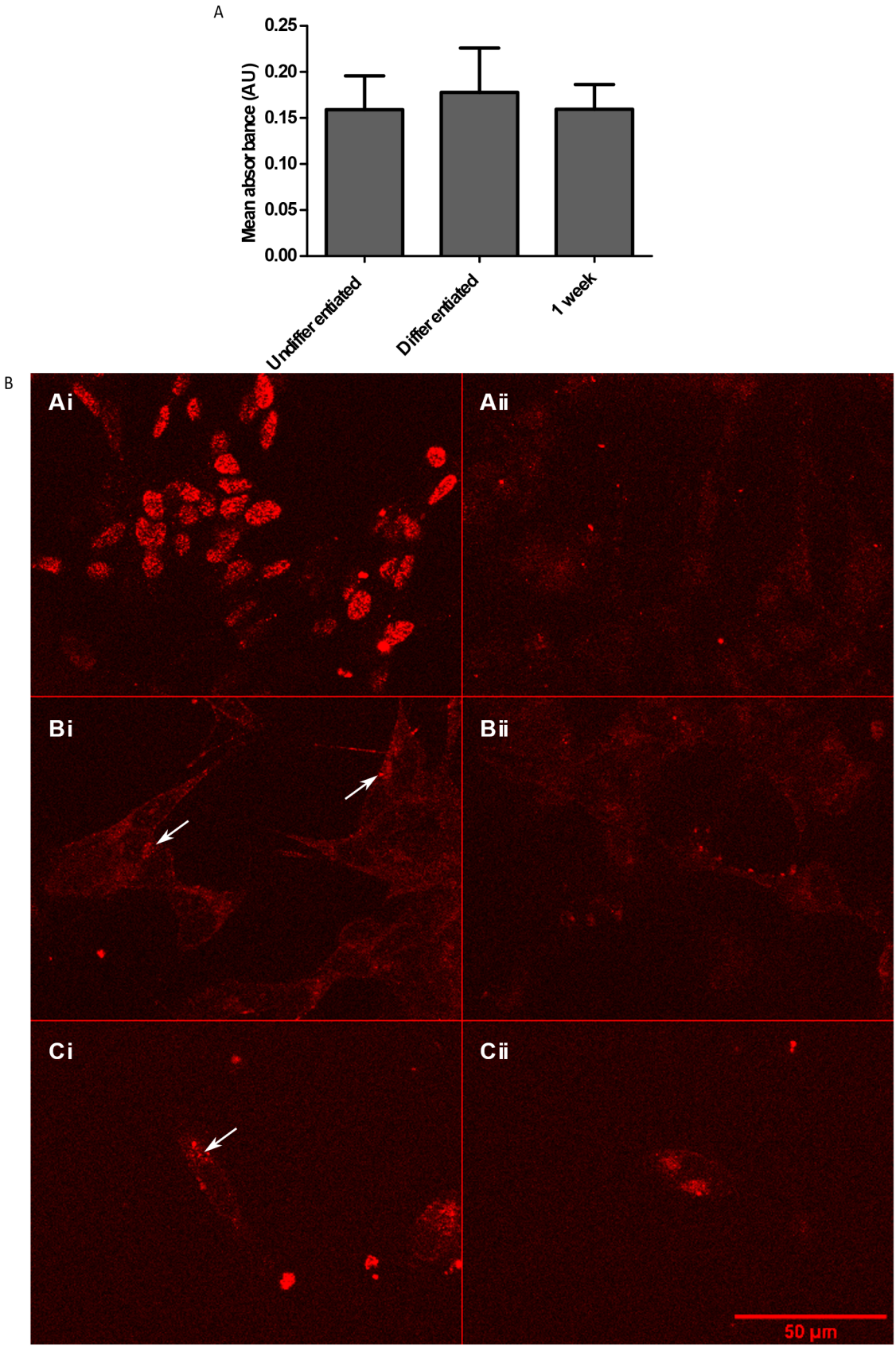

Figure 12. The leptin receptor is present in the undifferentiated and differentiated SHSY-5Y cell line.
A – The graph shows mean + SEM absorbance, as detected by ELISA, of ObR expression in

undifferentiated and differentiated SHSY-5Y cells. No difference in expression of the receptor was found ($H_{(2)}$ = 0.052, p=0.974, n=8) . B – Fluorescent images of undifferentiated (A), differentiated (B) and 1-week post differentiation (C) SHSY-5Y cell line are shown labelled with an ObR primary antibody and Texas red secondary (i) and labelled Texas red secondary in the absence of the primary antibody (ii). Greater staining in the primary containing conditions indicates the presence of the receptor in this cell line. White arrows indicate instances of ObR staining in the post-differentiation images.

The ELISA assay demonstrated that Ob-R is indeed expressed in this cell line at all stages of differentiation. No significant differences between the differentiation states was found (p=0.9744, n=8). Therefore, the levels of ObR expression did not change in SHSY-5Y cells during differentiation. This result was visually confirmed by representative ICC, SHSY-5Y cells were grown for up to 1-week post-differentiation and coverslips were fixed at 48h after plate down (undifferentiated, n=6), after 5 days in retinoic acid (differentiated, n=4) and after a further week in mitotic inhibitor medium (1 week, n=3). Staining was observed for all differentiation states but shows an interesting pattern where ObR expression is initially nuclear prior to induction of a neuronal phenotype. With differentiation, staining becomes cytoplasmic but little evidence of change is seen in expression patterns is observed thereafter.

5.3 LEPTIN$_{116-130}$ CAN PROTECT AGAINST NEURONAL DEATH IN VITRO, AND ITS EFFECTS ARE COMPARABLE TO THE FULL LEPTIN MOLECULE

The leptin hormone has been shown to be protective against induced cell death in primary cortical (Doherty *et al.*, 2013), dopaminergic midbrain and trigeminal sensory neurons (Doherty, Oldreive and Harvey, 2008). As such this demonstrates leptin is able to protect neurons in culture from cell death. To compare the neuroprotective abilities of leptin$_{116-130}$ to that of full-length leptin differentiated human neuroblastoma cells were treated with human, mouse and leptin$_{116-130}$ under toxic conditions. Given leptin$_{116-130}$ is a mouse sequence fragment both full-length mouse and human leptin were included in comparison for this test to ensure that mouse leptin was able to induce protective effects on SHSY-5Y cells and further that its effects were comparable to human leptin. This would ensure any effects of leptin$_{116-130}$ were due to its specific nature rather than a consequence of being a mouse fragment acting on human tissue. Circulating leptin levels have been reported between 0.3nM in lean subjects to 4nM in obese subjects and peaking at 100nM during development (Sinha *et*

al., 1996) therefore treatment concentrations were chosen in the range of 0.01-100nM in order to be physiologically relevant. To test the protective capabilities of these compounds, cells were treated with either copper chloride or amyloid beta in the following experiments.

5.3.1 Consequences of leptin exposure on neurotoxicity induced by copper chloride

Heavy metals have been implicated in many neurodegenerative diseases, for example in amyloid deposition in AD (Atwood *et al.*, 1998; Cherny *et al.*, 2001; Cavaleri, 2015), OS in Parkinson's disease (Sian-Hülsmann *et al.*, 2011; Kumar *et al.*, 2016) and protein misfolding in Amyotrophic Lateral Sclerosis (Sirangelo and Iannuzzi, 2017). As such, divalent metal ions have been widely used to model neurodegeneration *in vitro* (Hyun-jung Kim, 2005; Hirashima *et al.*, 2010; Isaev *et al.*, 2016). In this study cytotoxicity was induced with 10μM copper chloride and MTT and LDH assays were used to determine cell viability and representative photomicrographs were taken at selected concentrations for visual comparison after 96 hours.

5.3.1.1 Leptin does not prevent decreased mitochondrial activity associated with copper chloride exposure

An MTT assay is an indicator of cell viability by determining the level of mitochondrial activity. A higher absorbency reading indicated more functioning mitochondria present and hence greater cell viability (**Figure 13**).

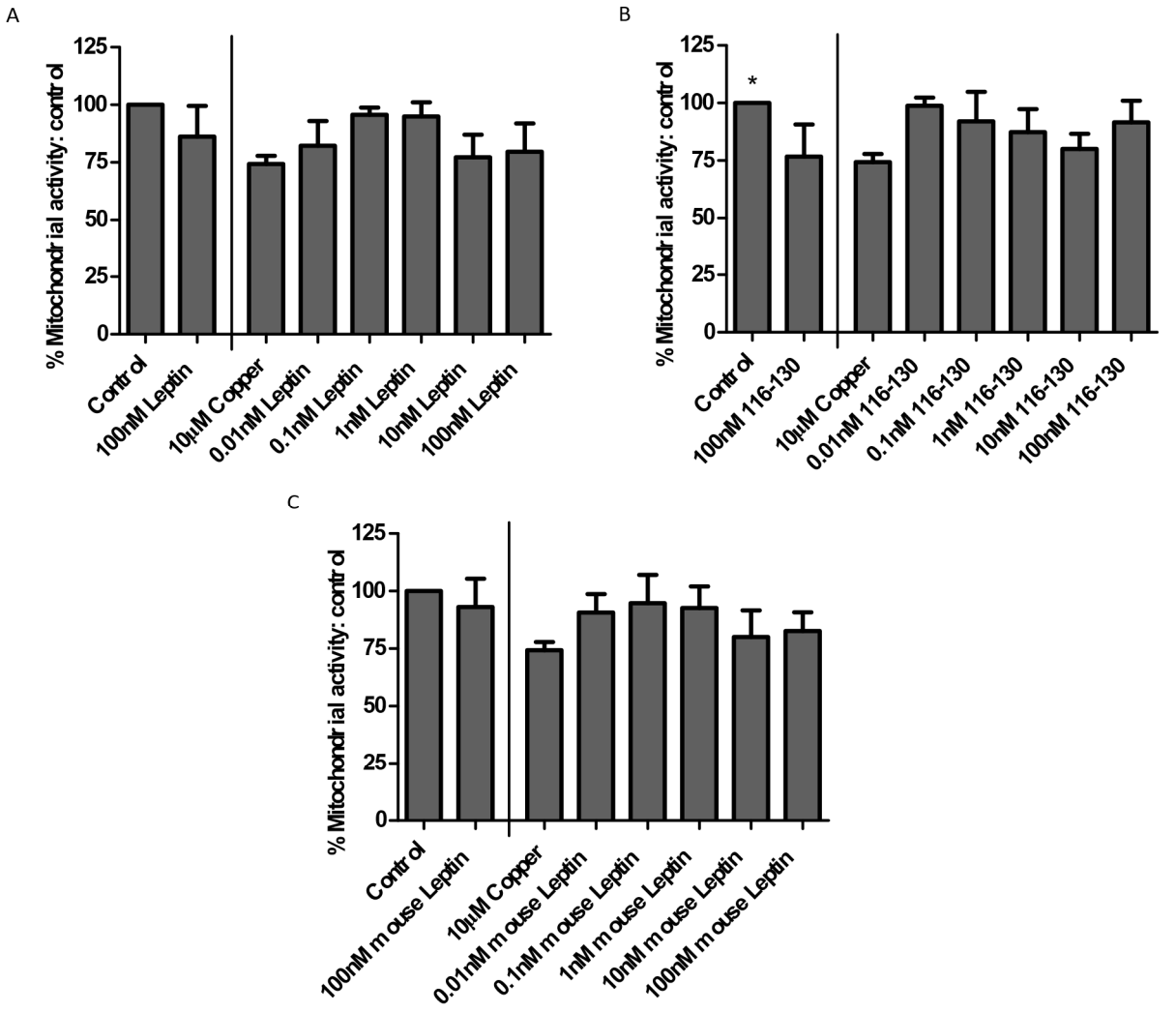

Figure 13 Leptin was unable to protect mitochondrial activity from the effects of copper toxicity. The graphs show mean + SEM mitochondrial activity relative to the untreated control for the range of leptin concentrations from 0.01-100nM used for three different leptin types: full length human (A; $H_{(7)}$=11.85, p=0.1055, n=6), fragment 116-130 (B; $H_{(7)}$=15.10, p=0.0347, n=6) and full-length mouse leptin (C; $F_{(7,40)}$=0.8925, p=0.5215, n=6) and treated with 10µM copper. The vertical line divides conditions without and with copper. Statistical significance relative to copper treated cells are denoted by an asterisk.

In order to determine if any of the three leptin types (human, mouse or 116-130) could produce a protective effect in this assay the data was split by leptin treatment and three separate analysis performed. Whilst a significant difference between untreated control and copper treatment was found in the $leptin_{116\text{-}130}$ data (untreated control had 25.73 ± 0% increase in MTT compared to copper treatment, $p<0.05$), no other significant differences were found. These results show that no concentration of any type of leptin was able to protect mitochondrial activity from copper toxicity.

5.3.1.2 Leptin prevents membrane rupture and lactate dehydrogenase release in response to copper chloride

An LDH assay indicates cell death by quantifying LDH release into the culture medium. LDH is an intracellular enzyme, however, during necrosis and late stage apoptosis the cell membrane ruptures and LDH is released into the medium. A higher absorbance in this assay indicates greater LDH release, which in turn suggests greater membrane rupture and therefore cell death (**Figure 14**).

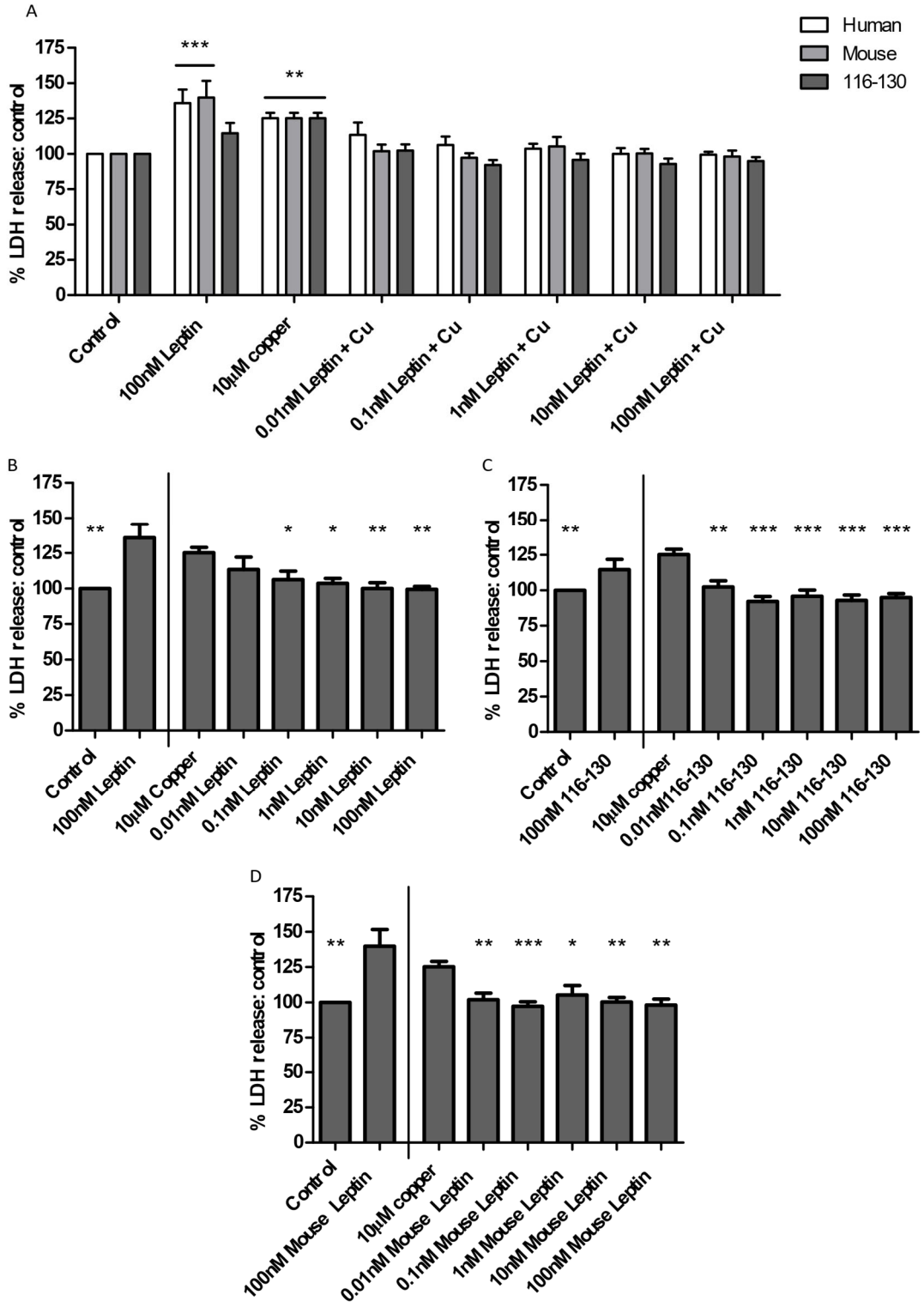

Figure 14. All forms of leptin were able to ameliorate increased LDH release caused by copper chloride treatment. The graphs show mean ± SEM LDH release relative to the untreated control for the range of leptin concentrations from 0.01-100nM used for three different leptin types. **A** – Graph shows all conditions on one axis to demonstrate differences between different concentrations of the three leptin types. Significance on this graph is relative to control. This data is then separated out into the three leptin preparations: Human leptin (**B**), $Leptin_{116\text{-}130}$ (**C**) and Mouse leptin (**D**) in order to more clearly demonstrate the dose response for each. In these three graphs significance is shown relative to the copper condition (leptin type: $F_{(2,163)}=5.334$, $p=0.0057$; Concentration: $F_{(7,163)}=18.50$, $p<0.0001$;

interaction: $F_{(14,163)}$=0.9257, p=0.5327; n=8) . A vertical line separates conditions without and with copper.

The control condition (25.15 ± 0%, p<0.01) and all concentrations of leptin treatments in the presence of copper, except 0.01nM human leptin, (Human leptin: 0.1nM = 18.93 ± 5.941% p<0.05, 1nM = 21.49 ± 3.509% p<0.05, 10nM = 25.14 ± 4.072% p<0.01 100nM = 25.80 ± 2.118% p<0.01; Mouse leptin: 0.01nM = 23.26 ± 4.646% p<0.01, 0.1nM = 27.98 ± 3.224% p<0.001, 1nM = 19.94 ± 6.725% p<0.05, 10nM = 24.83 ± 3.179% p<0.01, 100nM = 27.12 ± 4.207% p<0.01; $Leptin_{116-130}$: 0.01nM = 22.91 ± 4.408% p<0.01, 0.1nM = 33.09 ± 3.533% p<0.001, 1nM = 29.42 ± 4.456% p<0.001, 10nM = 32.29 ± 3.725% p<0.001, 100nM = 30.27 ± 2.776% p<0.001; n=8) had significantly decreased LDH release compared to copper alone. There was no significant difference between any leptin preparations demonstrating all concentrations protected similarly and neither human, mouse or $leptin_{116-130}$ showed difference in protective abilities. For leptin treatments without copper both 100nM human (35.84 ± % p<0.001) and 100nM mouse (39.84 ± % p<0.001) leptin had significantly increased LDH release relative to control but 100nM $leptin_{116-130}$ did not.

5.3.2 Consequences of leptin exposure on neurotoxicity induced by Aβ

Leptin has previously been shown to be protective against Aβ toxicity in primary cortical neurons (Doherty *et al.*, 2013), primary hippocampal neurons (Martins *et al.*, 2013), and a hypothalamic cell line (Gomes *et al.*, 2014). Hence to determine the ability of $leptin_{116-130}$ to protect against Aβ and see if it is comparable to leptin, differentiated SHSY-5Y cells were treated with full-length human leptin and $leptin_{116-130}$ over 96 hours with 10μM Aβ. In this study more refined parameters were used, as such concentrations of the leptins used were 0.1, 1 and 10nM and only human and $leptin_{116-130}$ were used. In the previous experiment viability was tested using MTT and LDH assays. However, the MTT data indicated no significant protective effect of leptin. Work in our lab has suggested that leptin can modulate oxidation via mitochondrial outer-membrane bound monoamine oxidase (MAO) A and B (Cheng *et al.*, no date). Other studies have also demonstrated leptin can increase mitochondrial oxygen consumption and biogenesis (Blanquer-Rosselló *et al.*, 2015), leptin deficient ob/ob mice showed

impaired electron transfer in the ETC (Munusamy *et al.*, 2015), and these mice had reduced mitochondrial respiration and expression of mitochondrial transcription factor in the liver which was ameliorated with leptin treatment but in extensor digitorum longus muscle leptin deficiency increased mitochondrial respiration (Holmström *et al.*, 2013). This demonstrates a role for leptin in the mitochondria which is tissue specific. As such the MTT assay may not be appropriate to assess cell viability with leptin treatments. Therefore, in these experiments LDH and crystal violet (CV) assays were used to determine cell viability instead (**Figure 15**). The CV assay is a measure of viable cell number, whereby higher absorbance readings indicate more viable cells. This data was gathered in collaboration with Dr G Doherty and D Redfearn for publication, thus presentation is as published (Malekizadeh *et al.*, 2017).

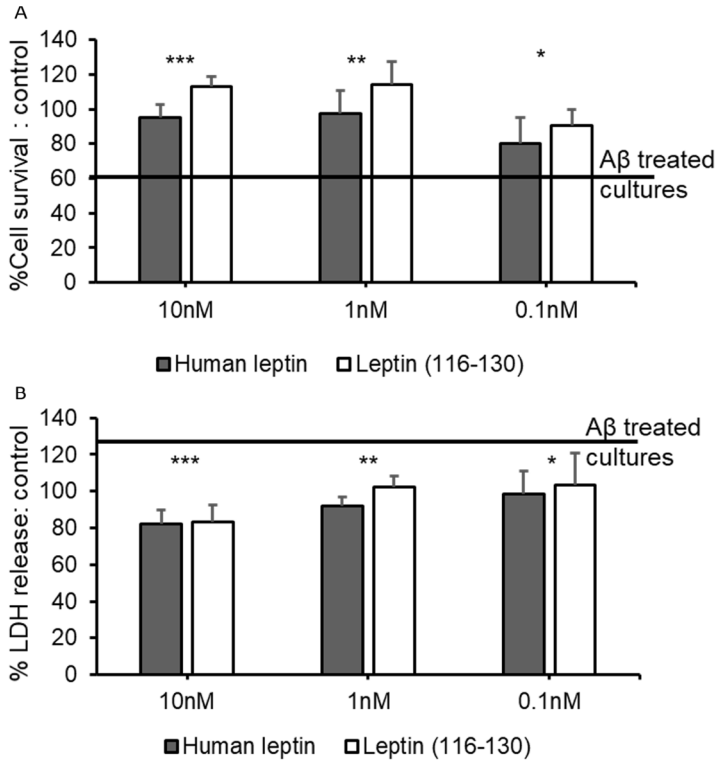

Figure 15. Leptin and Leptin$_{116-130}$ protect against the toxic effects of Aβ. Graphs show mean + SEM relative to untreated control. Horizontal line demonstrated mean of the Aβ treated cultures. A – Results from the crystal violet assay demonstrate all concentrations of leptin and leptin$_{116-130}$ had higher cell number compared to Aβ treatment alone. B – results of the LDH assay demonstrate all concentrations of leptin and leptin$_{116-130}$ had lower LDH release compared to Aβ treatment alone. Significance relative to Aβ treatment is denoted with asterisks.

The crystal violet assay demonstrated significant increases in cell number with both leptin and leptin$_{116-130}$ treatments relative to Aβ treated cultures (leptin: 10nM 44.3 ± 7.5%, $p<0.01$, 0.1nM 19.2 ± 15%, $p<0.05$; leptin$_{116-130}$: 10nM 51.8 ± 6.3%, $p<0.01$, 0.1nM 29.4 ± 9.4%, $p<0.05$; n=5). Further the LDH assay also demonstrated that all concentrations of both leptin and leptin$_{116-130}$ had significantly reduced LDH compared to Aβ treated cultures (leptin: 10nM 47.9 ± 7.45%, $p<0.01$, 0.1nM 31.8 ± 13.2%, $p<0.05$; leptin$_{116-130}$: 10nM 46.6 ± 9%%, $p<0.01$, 0.1nM 26.7 ± 17.3%, $p<0.05$; n=5). Therefore leptin$_{116-130}$, similarly to leptin, reduced cell loss and LDH release in response to AD-linked toxins *in vitro* (Malekizadeh *et al.*, 2017).

5.4 LEPTIN $_{116-130}$ ACTIVATES STAT3 AND AKT PRO-SURVIVAL PATHWAYS

Leptin has previously been shown to activate known cell survival pathways STAT3 and PI3K/Akt (Doherty *et al.*, 2013) and it is hypothesised that this is the mechanism by which it prevents neuronal cell death. As such in order to ascertain if leptin$_{116-130}$ activates the same pathways in its neuroprotection ELISA assay was carried out on differentiated SHSY-5Y cultures exposed to 1nM leptin$_{116-130}$ for 3 hours or left untreated prior to protein extraction (**Figure 16**). This data was gathered in collaboration with Dr G Doherty and D Redfearn for publication, thus presentation is as published (Malekizadeh *et al.*, 2017).

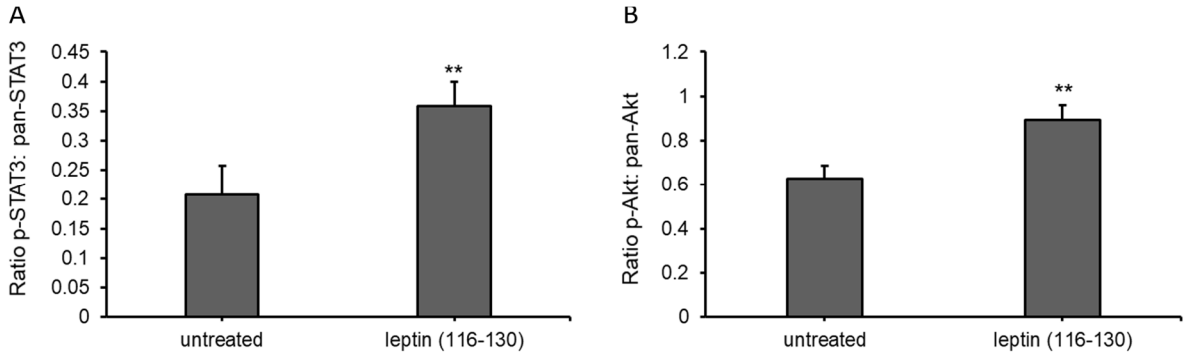

Figure 16. Leptin$_{116-130}$ activates STAT3 and Akt pathways to produce neuroprotective effects. Graphs show mean + SEM ratio of phosphorylated to pan signalling molecule as detected by ELISA assays. A – Shows the ratio of phosphorylated STAT3 to pan STAT3 in differentiated SHSY-5Y cells which are untreated or treated with 1nM leptin$_{116-130}$. B – shows the ratio of phosphorylated Akt to pan-Akt in untreated and leptin$_{116-130}$ treated SHSY-5Y cells. Significance relative to untreated control are denoted by asterisks.

T-tests showed the ratio of phosphorylated STAT3 to pan STAT3 was significantly increased by leptin$_{116-130}$ ($n = 3$; $p < 0.01$). Similarly, the ratio of phosphorylated Akt to pan Akt also increased following leptin$_{116-130}$ treatment ($n = 3$; $p< 0.01$). Thus leptin$_{116-130}$ can activate the STAT3 and PI3K/Akt pathways (Malekizadeh *et al.*, 2017).

5.5 LEPTIN AND LEPTIN$_{116-130}$ COULD NOT SIGNIFICANTLY MODULATE KNOWN BIOMARKERS OF ALZHEIMER'S DISEASE IN CULTURES TREATED WITH LOW-DOSE AB$_{1-42}$

In order to test whether leptin and leptin$_{116-130}$ could modulate biomarkers of AD, differentiated SHSY-5Y cultures were treated with 1µM Aβ_{1-42} for five days as well as 1nM leptin or leptin$_{116-130}$ with or without Aβ_{1-42}. Protein was then extracted from these cultures and Bradford assays run to determine equal loading. This concentration of Aβ was chosen because it has been previously shown to induce stress without inducing cell death over short time periods (Costa *et al.*, 2012; Gilson *et al.*, 2015). It was decided to treat cultures of five days as this was in line with the above viability test. The biomarkers that were chosen included tau and phosphorylated tau (p-tau), amyloid binding alcohol dehydrogenase (ABAD), amyloid precursor protein (APP), endophilin 1 and presenilin 1. These were chosen because they are considered the main biomarkers of the disease correlating with cognitive impairment due to AD (p-tau; Olsson *et al.*, 2016) or integral to its pathology (ABAD (Borger *et al.*, 2013), APP (Dawkins and Small, 2014), endophilin 1 (Ren *et al.*, 2008), presenilin 1 (Somavarapu and Kepp, 2016)) . The p-tau used was phosphorylated at serine 262 because this is one of the earliest sites to become phosphorylated in the AD brain (Augustinack *et al.*, 2002) and is a required site for tangle pathology (Wang, Grundke-Iqbal and Iqbal, 2007). Although 8 experimental repeats were made only an n of 3 were used in these experiments. This is because previous literature has shown that Aβ increases p-tau (Manassero *et al.*, 2016; Bennett *et al.*, 2017), as such any cultures where p-tau was not increased were not tested further for other biomarkers.

5.5.1 ELISA assay was unable to detect any significant changes in AD biomarkers generated by low-dose Aβ or modulation by leptin or leptin$_{116-130}$

The ELISA assay allows exploration of the types and relative levels of proteins present in a sample by tagging them with antibodies specific to the protein of interest. This can then be tagged in turn with a conjugated secondary which will react with a substrate to produce a colorimetric output. The absorbency of this can be read and analysed. In this assay a higher absorbance indicates a greater binding of the primary antibody and therefore more of the target protein present within a sample. In these ELISA assays the following antibodies were used (see **Table 3** for concentrations): p-tau, tau, ABAD, APP, endophilin 1 and presenilin 1. α-tubulin was used as a loading control. LDH assays were also performed on these cultures and the results of control and 1μM Aβ treatments are shown (**Figure 17**).

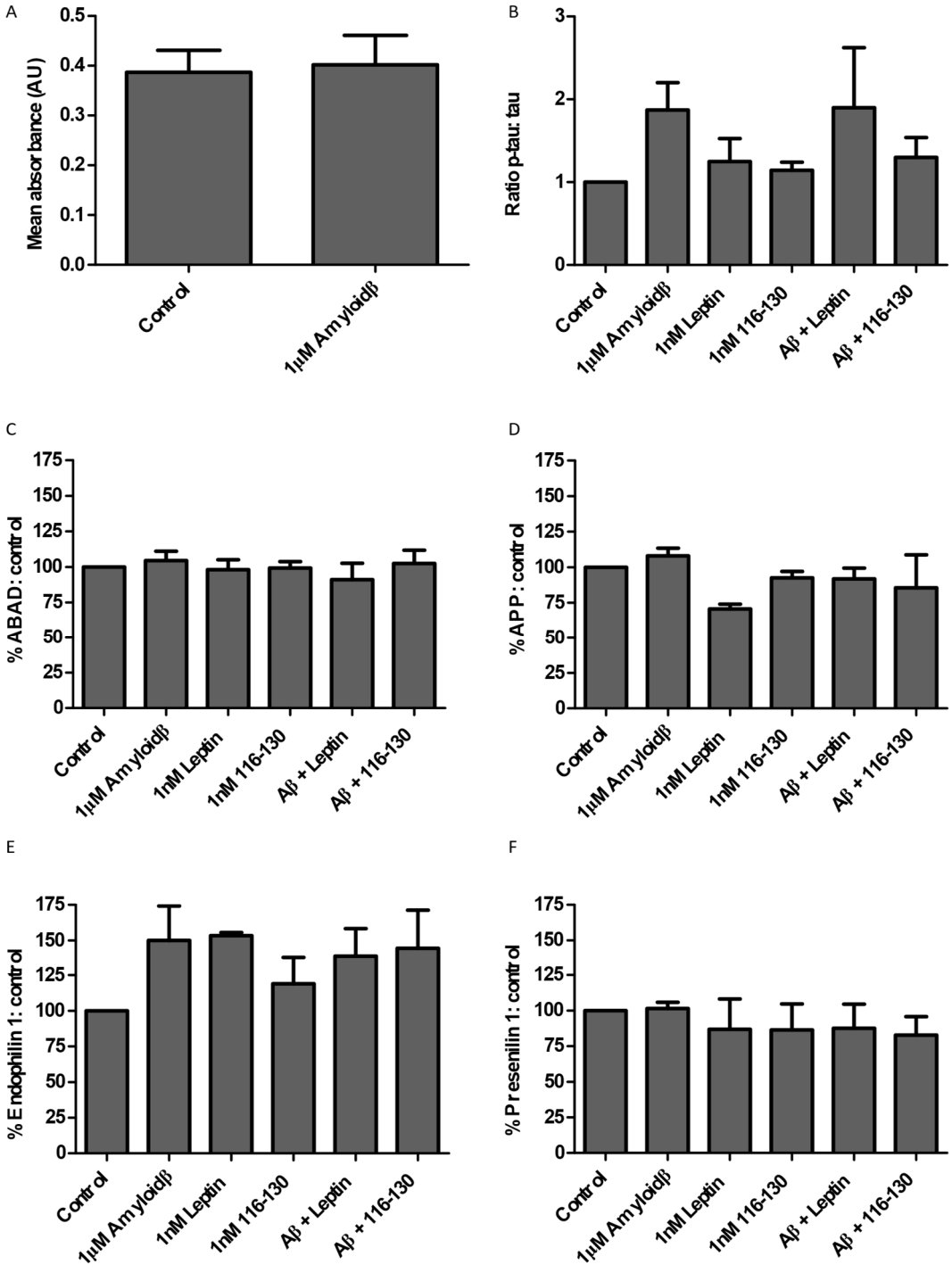

Figure 17. ELISA for AD biomarkers in cultures treated with Aβ, leptin and leptin$_{116-130}$. The graphs show mean + SEM. Within the graphs the following abbreviations are used Aβ for amyloid beta and 116-130 for leptin$_{116-130}$. A – Shows the results for an LDH assay on control and 1μM amyloid beta treated cells showing Aβ did not induce cell death in these cultures ($t_{(5)}$=0.8454 p=0.4365 n=3) B – Shows results from p-tau and tau ELISAs as a ratio. Aβ appears to have increased p-tau relative to tau and leptin is unable to ameliorate this but leptin$_{116-130}$ does although this did not reach significance ($H_{(5)}$=6.195 p=0.2877, n=3) . C – Shows no variation in ABAD levels with any treatment ($F_{(5,12)}$=0.3823 p=0.8514, n=3). D – There is a trend towards decreased APP with 1nM leptin alone however this is not sustained in combination with Aβ ($F_{(5,12)}$=0.3832 p=0.2637, n=3). E – All treatments show a suggested increase in endophilin 1 relative to control ($F_{(5,12)}$=1.248 p=0.3467, n=3). F – Shows there may be a

trend towards decreased presenilin 1 levels with both leptins with and without Aβ but no difference between control and Aβ ($F_{(5,12)}$=0.2916 p=0.9086, n=3).

No significant differences were found between control and Aβ in the LDH assay (p= 0.4365) or any treatments for the biomarkers tested (p-tau: tau p=0.2877; ABAD p=0.8514; APP p=0.2637; endophilin 1 p=0.3467; presenilin 1 p=0.9086). These data indicate that the Aβ treatment used did not induce cell death and neither leptin nor $leptin_{116-130}$ were able to modulate AD biomarkers in these cultures, however it also indicates that Aβ could not significantly upregulate the biomarkers in these cultures either.

5.6 LEPTIN AND $LEPTIN_{116-130}$ CAN ACT AS COGNITIVE ENHANCERS IN AN EPISODIC-LIKE MEMORY TASK.

Given $leptin_{116-130}$ was able to act as a neuroprotective with equivalent ability to leptin and leptin has previously been shown to be beneficial to memory (X-L Li *et al.*, 2002; Valladolid-Acebes *et al.*, 2013), the potential of these molecules to modulate episodic memory was assessed. Following the administration of a vehicle control, leptin or $leptin_{116-130}$ mice were assessed on the OPC memory task to establish changes in their episodic-like memory.

Episodic memory loss is one of the earliest cognitive deficits in AD (Tromp *et al.*, 2015). As we age a natural decline in memory is observed which may sometimes be defined as mild cognitive impairment (MCI) however this degree of memory loss is not necessarily an indicator of AD (Behrman, Valkanova and Allan, 2017). To be given a diagnosis of AD a patient must demonstrate not only cognitive decline on objective measures but do so to a degree which interferes with daily life and independence (Knopman and Petersen, 2014). Currently more sensitive episodic memory tests are being developed which may be able to detect differences between patients who present with MCI and will decline no further and those who will convert to AD (Marra *et al.*, 2016). As such an effective AD treatment must be able to tackle episodic memory decline in order to have life-enhancing effects for the patients.

To test if a potential therapeutic could counteract cognitive decline in a manner relevant to AD a test of episodic memory in animal models must be used. An episodic memory requires the recall of the

what, where and when components of a specific event (Eacott and Easton, 2010; Easton, Webster and Eacott, 2012) and requires the ability of mental time travel to reconstruct personal events. There has been long debate about the ability of animals to do this (Suddendorf and Corballis, 1997; Corballis, 2013) however, evidence showing that animals can integrate what-where-which events and that this integration occurs within the hippocampus (Eacott and Norman, 2004; Langston and Wood, 2009; Wilson, Watanabe, *et al.*, 2013) has led to the conclusion that some animals are capable of episodic-like memory. This can be tested using the object-place-context (OPC) recognition task as it combines all three of the what-where-which components of episodic-like memory.

5.6.1 All pre-treatment groups performed similarly on the NOR, OP and OC task

Mice in all groups performed NOR, OP and OC recognition tasks pre-treatment to ensure they were able to perform the component parts of the OPC task. This allowed for assessment of their abilities pre-treatment (Figure 18). Any trial where a mouse explored for a total time of less than 5 seconds was removed from final analysis. After completing these tasks but before analysis of this data mice were assigned to their treatment groups in accordance with counterbalancing.

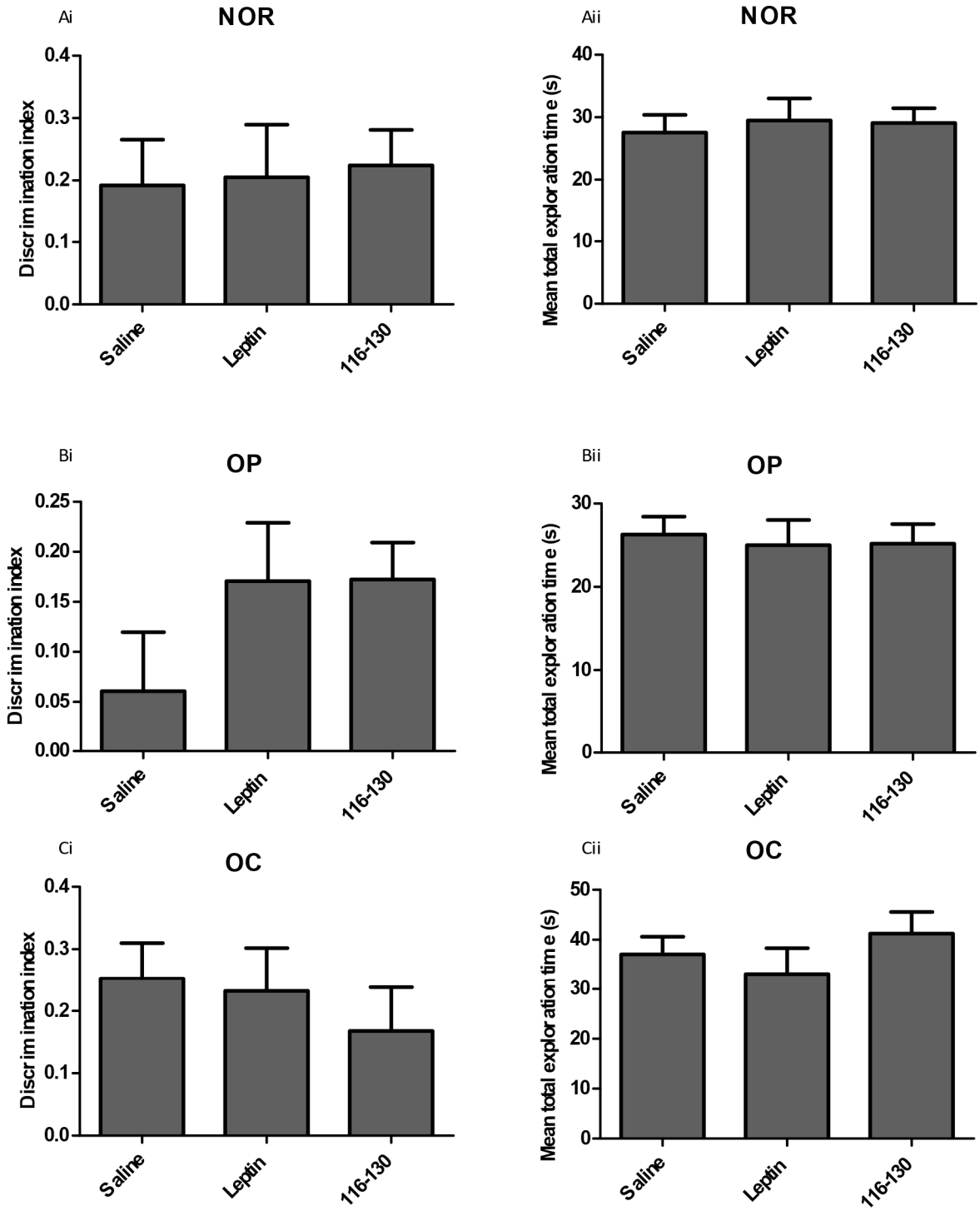

Figure 18. All pre-treatment groups of mice performed similarly on NOR, OP and OC tasks. All graphs show mean + SEM. A – performance on the NOR task measured via discrimination index ($F_{(2,38)}$=0.0491 p=0.9522, n=14) (i) and total exploration time ($H_{(2)}$=0.3518 p=0.8387, n=14) (ii). B – performance on the OP task measured via discrimination index ($F_{(2,38)}$=1.466 p=0.2436, n=14) (i) and total exploration time($F_{(2,38)}$=0.0742 p=0.9287, n=14) (ii). C- performance on the OC task measured via discrimination index ($F_{(2,39)}$=0.4502 p=0.6408, n=14) (i) and total exploration time ($F_{(2,39)}$=0.8541 p=0.4335, n=14) (ii).

In the NOR there was no significant difference between pre-treatment groups discrimination index (d.i) scores (p=0.9522). One sample t-tests against theoretical mean of 0, showing no preference for either novel or familiar object, showed all animals performed above chance levels (saline $t_{(12)}$=2.626, p=0.0222; leptin $t_{(13)}$=2.430, p=0.0303; 116-130 $t_{(13)}$=3.934, p=0.0017). Further, the mean exploration time data showed no significant difference between groups (p=0.8387). This shows all animals were able to discriminate between novel and familiar objects with a preference for novel objects and explored similarly.

In the OP task there was no significant difference between the groups d.i. scores (p=0.2436) but the leptin and $leptin_{116\text{-}130}$ pre-treatment groups performed above chance (leptin $t_{(13)}$=2.923, p=0.0119; 116-130 $t_{(12)}$=4.674, p=0.0005) whereas the saline group did not ($t_{(13)}$=1.029, p=0.3222). There was no difference in mean exploration time between groups (p=0.9287) This shows that although all pre-treatment groups performed and explored similarly on this task the saline group did not perform above chance in discriminating between novel and familiar objects when place was the key factor.

In the OC task there was no difference between groups d.i. (p=0.6408.), all animals performed above chance (saline $t_{(13)}$=4.433, p=0.0007; leptin $t_{(13)}$=3.325, p=0.0055; 116-130 $t_{(13)}$=2.383, p=0.0331) and there was no significant difference in mean exploration time between groups (p=0.4335). This shows all pre-treatment groups were able to discriminate a novel object in a context and explored similarly across groups.

In order to further explore the lack of above chance performance in the pre-treatment saline group on the OP task further analysis was conducted on the discrimination index scores for each OP trial and how the placement of the object may have affected their ability to perform this task (**Figure 19**).

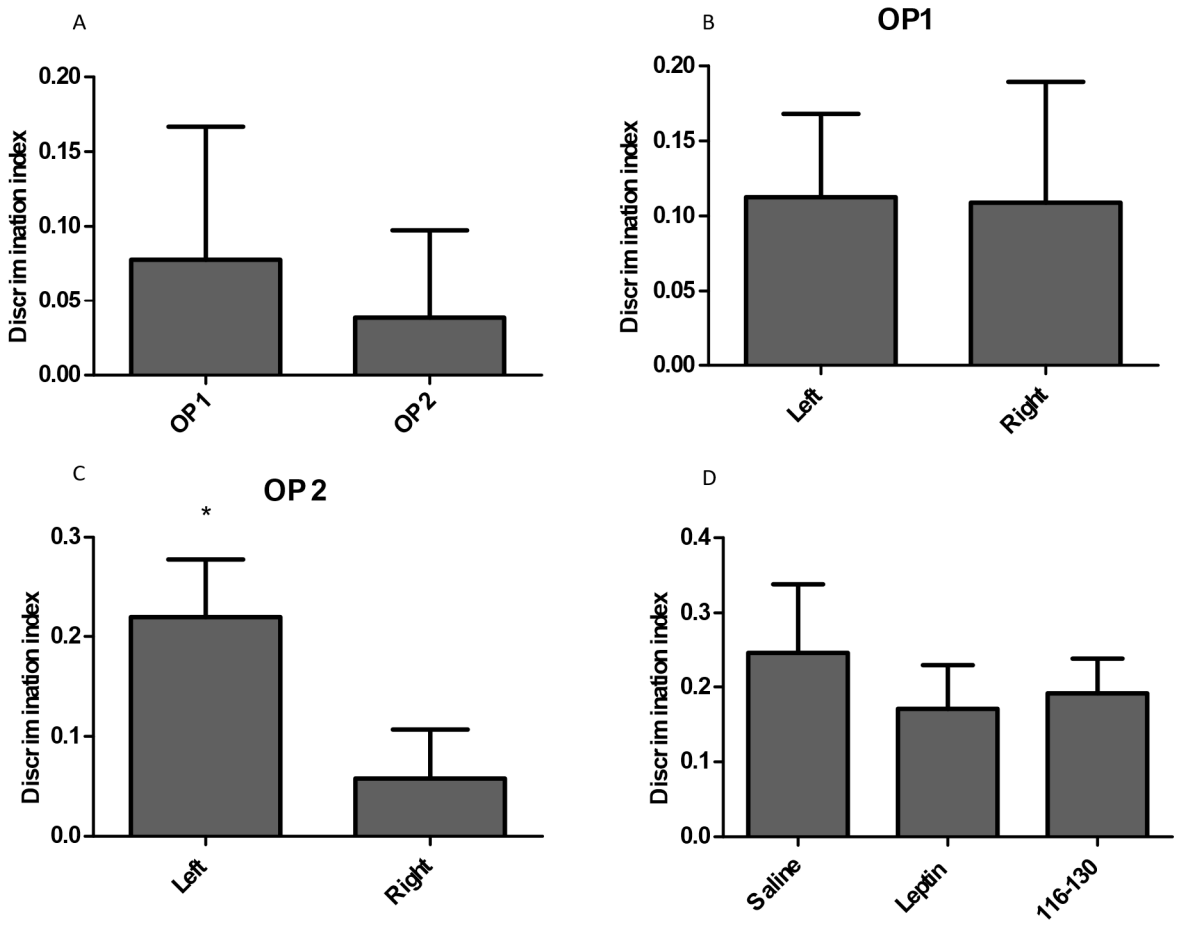

Figure 19. Exploration of the pre-treatment saline groups inability to perform above chance on the OP task. **A**- discrimination index scores on first and second trials of the OP task (OP1 and OP2 respectively) for pre-treatment saline mice ($t_{(24)}$=0.367, p=0.7168, n= 14) . **B** – discrimination index in OP1 for all mice when the novel object was positioned on the left and right ($t_{(37)}$=0.0367, p=0.7168, n=21). **C** – discrimination index in OP2 for all mice when the novel object was positioned on the left and right ($t_{(38)}$=2.131, p=0.0396, n=21). **D** – discrimination index averaged across OP1 and OP2 with trials removed where mice explored less than 5 seconds in the sample phase ($F_{(2,38)}$=0.3206, p=0.7277, n=14) .

The d.i. data for the pre-treatment saline group was assessed for each trial in the OP task (**Figure 19**A; trial 1 = OP1, trial 2 = OP2) although a t-test demonstrated no significant difference between trials (p=0.1674) there was a trend towards lower scores in OP2. The d.i. data for all mice based on the novel objects position (left or right relative to the mouse's starting position) was assessed for both trials (**Figure 19**B OP1; **Figure 19**C OP2). No significant difference was found in OP1 (p=0.9710) but there was a significant difference in OP2 (p=0.0396). This shows that across all mice the identification of the novel object in place was significantly better when the novel object was on the left in the second trial. Mice in the pre-treatment saline group in OP2 saw the novel object more frequently

on the right (9 times) than the left (6 times) which may help explain their reduced discrimination index score on this test. Finally time spent exploring objects during the sample phases were assessed and any trial where the mouse explored for less than 5 seconds in either sample phase was removed and the average d.i. score recalculated. This data shows no significant difference between d.i. scores of the pre-treatment saline, leptin or $leptin_{116-130}$ groups and all groups now demonstrate above chance performance in this task (saline: $t_{(12)}=2.643$, $p=0.0214$; leptin: $t_{(13)}=2.923$, $p=0.0119$; $leptin_{116-130}$: $t_{(12)}=4.139$, $p=0.0012$). Therefore it can be concluded that lack of exploration in the sample phase decreased encoding of the object-place association.

5.6.2 Leptin and $Leptin_{116-130}$ improve performance on an OPC task

After completing all trials of NOR, OP and OC testing mice were tested on the OPC task. Mice were injected intraperitoneally with saline, leptin or $leptin_{116-130}$ (7.8nM/ml, based on concentrations used previously (Grasso *et al.*, 1997, 2001; Rozhavskaya-Arena *et al.*, 2000)) 30 minutes prior to their testing time to allow time for the drug to circulate through their system and to allow for recovery from injection. Their performance on the task was assessed post-testing and their discrimination index and total exploration times recorded at both 1 and 3minute time points (**Figure 20**).

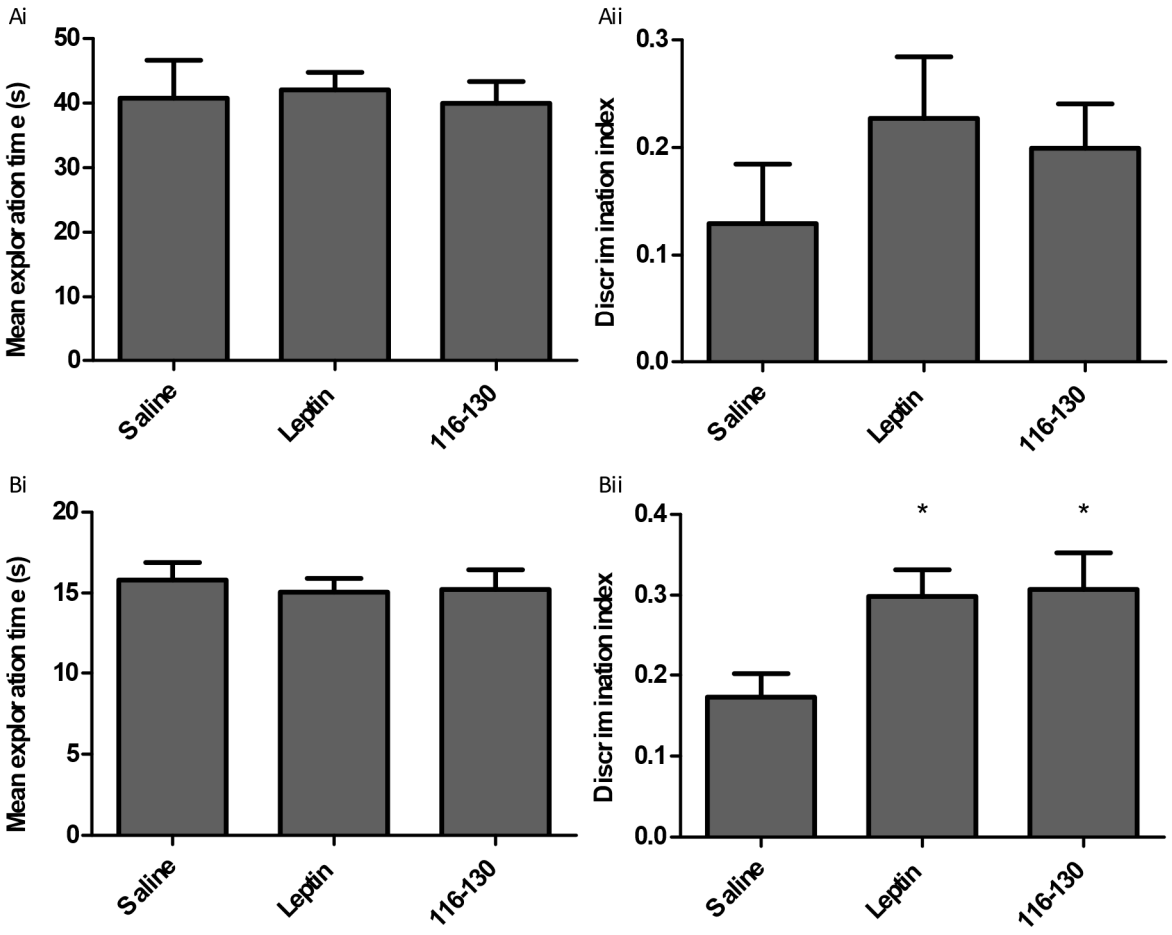

Figure 20. Leptin and Leptin$_{116-130}$ were able to improve performance of mice on an episodic-like memory task. All bar graphs show mean + SEM Ai – The graph shows the exploration times of the mice for the OPC task at the 3-minute time point ($F_{(2,39)}$=0.0649, p=0.9375, n=14) . Aii – Shows the discrimination index for the three groups at the 3-minute time point, all groups performed above chance but there was no difference between them ($F_{(2,39)}$=0.9450, p=0.3974, n=14). Bi – The graph shows exploration time in seconds of mice on the object-place-context recognition task. There was no difference between the total exploration times of each group of mice ($F_{(2,39)}$=0.1428, p=0.8674, n=14). Bii - The graph shows the discrimination index for the three groups. Both Leptin and leptin$_{116-130}$ treated mice had a significantly greater discrimination index compared to control ($H_{(2)}$=8.898, p=0.0117, n=14). Significance relative to saline treated control is denoted by an asterisk.

At the full 3-minute time point the data showed no significant difference between groups exploration time (p=0.9375) or d.i. score (p=0.3974). One-sample t-tests to hypothetical mean of 0 showed all groups performed above chance (saline $t_{(13)}$=2.334, p=0.0363, n=14; leptin $t_{(13)}$=3.965, p=0.0016, n=14; 116-130 $t_{(13)}$=4.806, p=0.0003, n=14). This shows that all mice performed above chance in this task but there was no significant difference between groups.

It has previously been shown that preference for the novel object can significantly reduce over a trial phase with peak interest occurring within the first few seconds (Clark, Zola and Squire, 2000) as such a cut off of 1 minute was agreed to capture the time period in which mice are most likely to hold interest in novelty. Therefore, data after 1 minute of the OPC test phase was also analysed. The 1-minute discrimination index data showed that the leptin (0.1266 ± 0.0329 a.u., $p<0.05$) and leptin$_{116\text{-}130}$ treated mice (0.2061 ± 0.0452 a.u., $p<0.05$) had significantly higher d.i. score than the saline group. There was no significant difference between the leptin and the leptin$_{116\text{-}130}$ treated mice. Wilcoxon signed rank tests to hypothetical median of 0 demonstrated all groups performed above chance (saline $W_{(12)}=91$, $p=0.0016$, $n=13$; leptin $W_{(13)}=105$, $p=0.0001$, $n=14$; 116-130 $W_{(13)}=105$, $p=0.0011$, $n=14$). There was no significant difference between the mean exploration times of the different groups ($p=0.8674$). This demonstrates all mice were able to perform this task and further the leptin and leptin$_{116\text{-}130}$ treatment groups were able to perform significantly better than control and hence suggests a memory enhancing effect for these drugs.

5.6.3 Neither leptin nor leptin$_{116\text{-}130}$ had a significant impact on mouse weight over the course of testing

As leptin is a satiety inducing hormone it was important to assess if treatment with leptin and leptin$_{116\text{-}130}$ had an impact on the weight of the mice over the course of the memory experiment. As such mice were weighed daily over the course of the OPC trials in order to assess any weight changes (Figure 21).

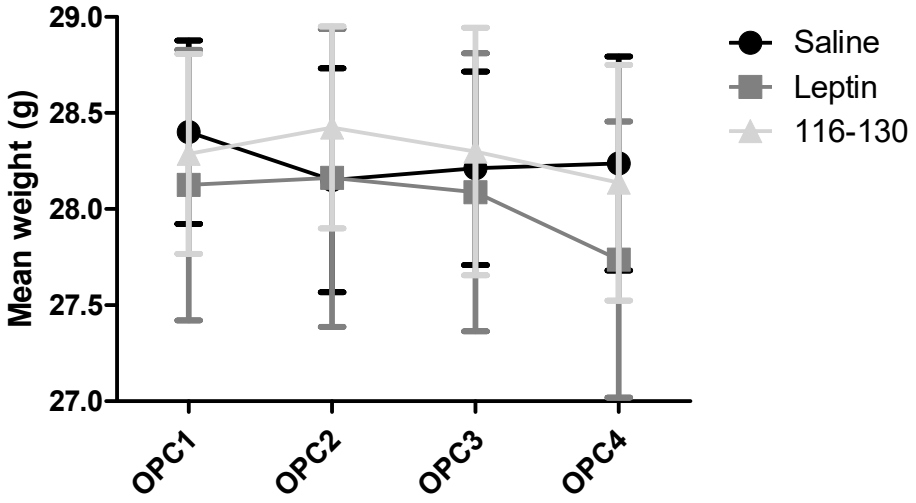

Figure 21. There was no significant change in mouse weight over the course of injections in any treatment group. The line graph shows mean +/- SEM weight for saline, leptin and leptin$_{116\text{-}130}$ treated mice on each day of the OPC task (OPC number: $F_{(3,63)}$=2.145, p=0.1034; Treatment: $F_{(2,63)}$=0.0528, p=0.9487; Subjects (matching): $F_{(21,63)}$=96.78, p<0.0001; Interaction: $F_{(6,63)}$=0.9973, p=0.4351; n=8).

No significant difference was seen in treatment (p=0.9487), OPC trial (p=0.1034) or their interaction (p=0.4351). Weight did not change significantly across the four trials for any treatment (saline, leptin or leptin$_{116\text{-}130}$) and there were no differences between treatment groups weights on any day. Therefore, neither leptin nor leptin$_{116\text{-}130}$ had an impact on the weight of the mice relative to the saline group.

5.6.4 Protein extracted from brains of mice after OPC memory task did not show any long-term changes in ObR expression or leptin level expression by ELISA

The OPC task was carried out on two separate batches of mice. Following completion of the OPC task by the second batch of mice the animals were culled by cervical dislocation in accordance with Schedule 1 of UK Animals (Scientific Procedures) Act, 1986 and their brains extracted for assessment of ObR expression and total leptin levels by ELISA (Figure 22). Protein was extracted from one hemisphere from each mouse. ObR expression was detected using the in-house ELISA method (ObR primary antibody (2 wells) and α-tubulin (1 well) as a loading control, and mouse secondary antibody, see **Table 3**) (**Figure 22**A). Total leptin brain levels were elucidated using a commercially available kit which can

detect mouse leptin from amino acids 22-167, and total levels were compared to individual performance on the OPC task (**Figure 22**B-F).

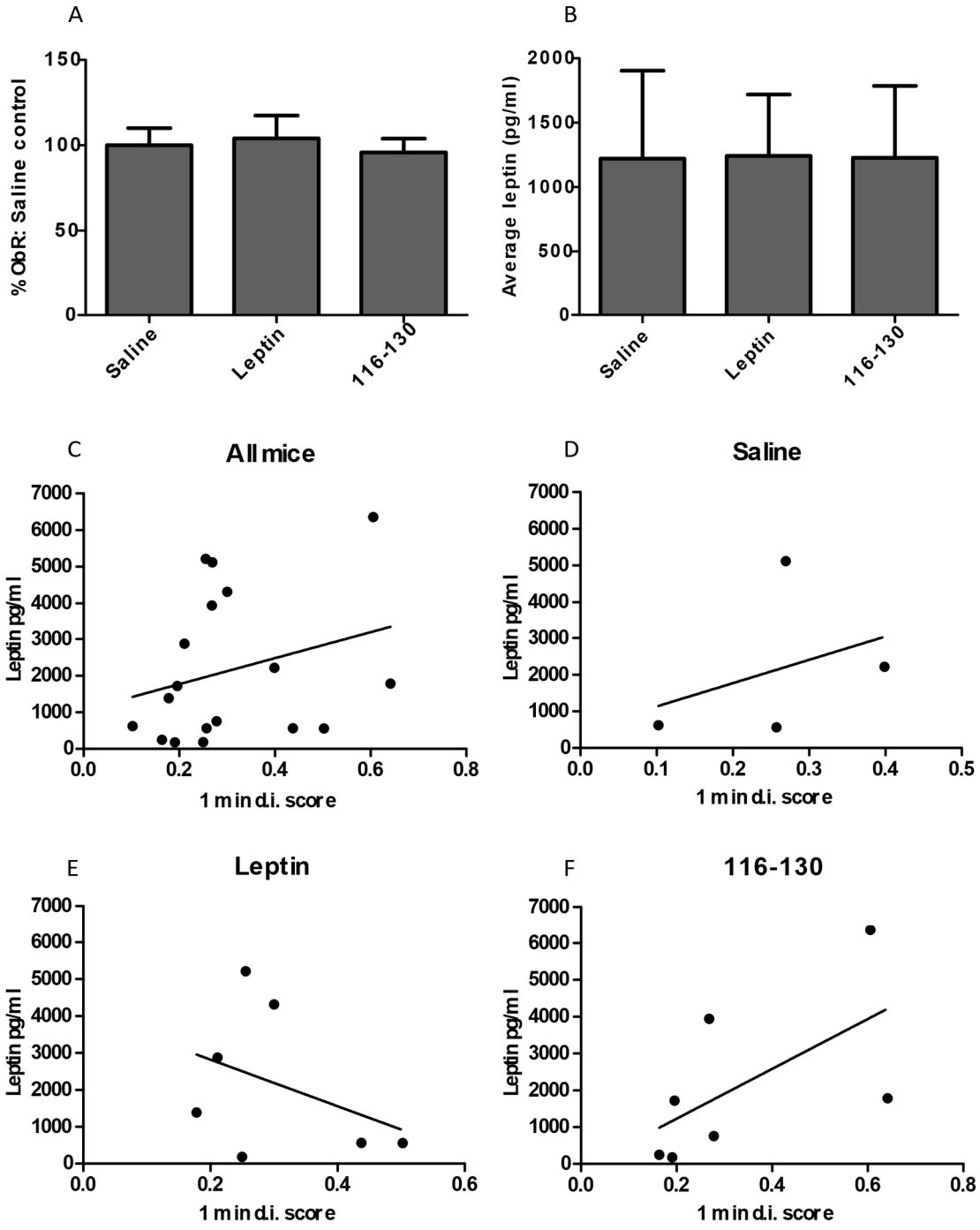

Figure 22. Mice treated with leptin or leptin$_{116-130}$ showed no changes in ObR expression or absolute leptin levels in the brain relative to mice treated with saline after OPC memory task. A – The graph shows mean + SEM %ObR expression relative to the saline treated control mice as detected by ELISA. There was no difference in ObR expression between the groups ($F_{(2,21)}$=0.1458, p=0.8652, n=8). B - The graph shows mean + SEM absolute leptin concentrations (pg/ml) as detected by ELISA. There was no difference in leptin levels between treatment groups ($H_{(2)}$=0.3346, p=0.8459, n=7). C – Shows the leptin levels (pg/ml) of each mouse relative to their discrimination index on the OPC task scored for 1

minute ($F_{(1,16)}$=1.241, R^2 = 0.07, p = 0.28, n=18) which is then broken down into the three treatment groups: Saline (D; $F_{(1,2)}$=0.3008, R^2 = 0.13, p = 0.64, n=4), Leptin (E; $F_{(1,5)}$=0.8413, R^2 = 0.14, p = 0.40, n=7) and $Leptin_{116-130}$ (F; $F_{(1,5)}$=2.875 R^2 = 0.37, p = 0.15, n=7).

There was no significant difference in ObR expression (p= 0.8652) or leptin levels (p=0.8459) in the brain between the treatment groups (saline n=4, leptin n=7, $leptin_{116-130}$ n=7). Therefore, treatment with leptin or $leptin_{116-130}$ did not affect expression of the receptor or leptin levels in the brains of these mice. Although averaged across the groups these mice were able to perform the OPC task and those in either leptin or $leptin_{116-130}$ treatment group performed significantly better than saline control not all mice performed equally. It is possible those that performed better on the task did so because of differences in leptin uptake or release, as such the performance on the OPC task was compared to the brain leptin levels to identify any correlations **(Figure 22**C-F). Assessed either as a group (R^2 =0.07196, p=0.2818), or by individual treatment (saline: R^2=0.1308, p=0.6384; leptin R^2 = 0.1440, p=0.4011; and $leptin_{116-130}$ R^2=0.3651, p=0.1507), no significant correlations were found. This shows that treatment of these mice with leptin or $leptin_{116-130}$ did not upregulate the ObR receptor or leptin levels long-term in the murine brain, further there was no correlation to actual brain leptin levels and discrimination index score in these mice.

5.7 LEPTIN AND $LEPTIN_{116-130}$ COULD NOT PROMOTE HT22 SURVIVAL IN A SERUM FREE ENVIRONMENT.

Given that leptin and $leptin_{116-130}$ demonstrated protective effects in SHSY-5Y neurons which tend to be cholinergic (Kovalevich and Langford, 2013) and they can act as cognitive enhancers in healthy mice, the investigation of their protective ability in a murine hippocampal cell line (HT22) was carried out. Undifferentiated HT22 cells were used in this pilot study and initially different methods of inducing cell death were tested via CV assay (**Figure 23**). The kill conditions chosen where 5mM glutamate application and serum reduction over 24 h as these have previously been suggested to induce cell death in this model (Rössler *et al.*, 2004; Kulawiak and Szewczyk, 2012).

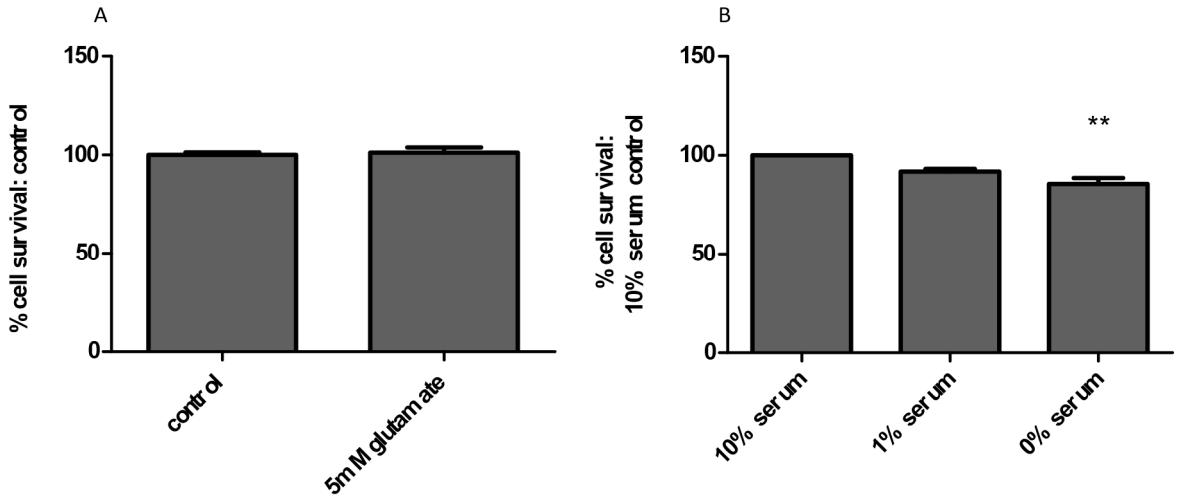

Figure 23 Serum starvation but not glutamate treatment over 24h was able to induce cell death in HT22 cells. Graphs show mean +SEM cell survival as measured by crystal violet assay. **A** – HT22 cells treated for 24h with 5mM glutamate showed no significant changes in cell survival compared to the control population ($t_{(20)}$=0.6361, p=0.7203, n=10) . **B** – HT22 cells treated for 24h with 0% serum but not 1% serum showed significant decrease in cell survival relative to 10% serum control ($H_{(2)}$=10.89, p=0.043, n=5).

No significant difference between control and 5mM glutamate treated cells (p=0.7203) was found. However, in the serum reduction trial significantly reduced cell survival occurred between 10% and 0% serum conditions (14.48 ± 3.059, p<0.01) but not between 10% and 1% or 1% and 0% conditions (p>0.05 for both). These data indicate that complete serum removal but not serum reduction (1%) or glutamate treatment could induce cell death in HT22 cells.

Given these findings 0% serum was chosen as the inducer of cell death to test a range of leptin and leptin$_{116\text{-}130}$ concentrations from 1-100nM as potential protective agents in HT22 cells using LDH and CV assays (Figure 24).

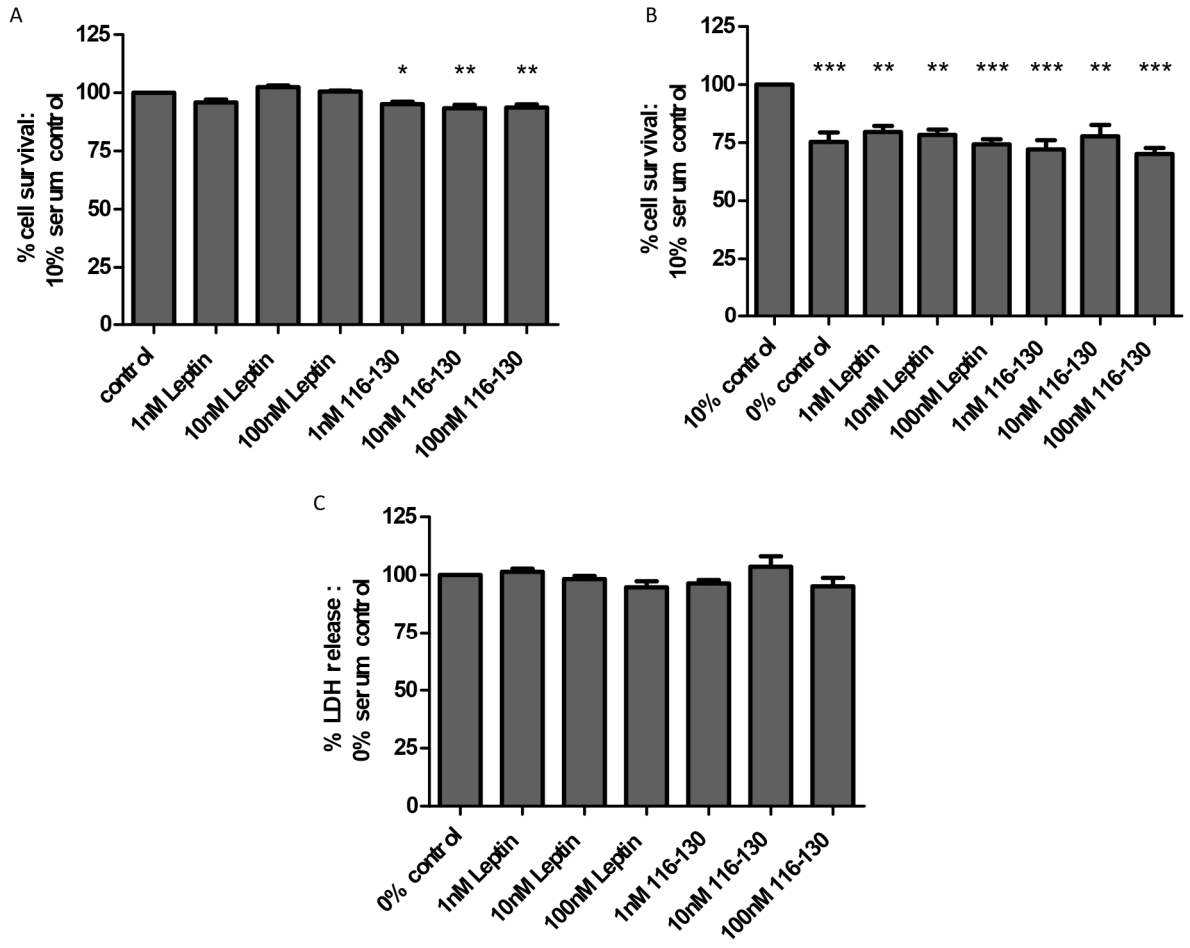

Figure 24. Neither treatment with leptin nor leptin$_{116-130}$ can protect HT22 cells from full serum deprivation. A- The graph shows mean + SEM % cell survival relative to the 10% serum control cells as determined by crystal violet assay for leptin and leptin$_{116-130}$ treatments under 10% control conditions. Small but significant decreases in cell number were found in all leptin$_{116-130}$ conditions ($F_{(6,14)}$=12.47, p<0.0001, n=3) . B – Graph shows mean + SEM % cell survival relative to the 10% serum control as determined by crystal violet assay for 0% serum containing conditions. All conditions lacking serum showed significant decrease in cell number relative to the 10% control ($F_{(7,16)}$=8.568, p=0.0002, n=3). C - The graph shows mean + SEM % LDH release relative to 0% serum control. No significant difference was found between conditions ($F_{(6,14)}$=1.712, p=0.1906, n=3). Significance is denoted by asterisks.

In the crystal violet assay data (n=3) showed under 10% serum conditions all concentrations of leptin$_{116-130}$ had significantly reduced cell survival compared to the 10% control (1nM 4.867 ± 1.068%, p<0.05; 10nM 6.644 ± 1.453%, p<0.01; 100nM 6.321 ± 1.366%, p<0.01) and under 0% serum conditions all treatments had significantly reduced cell survival relative to 10% (0% serum 24.65 ± 4.025%, p<0.001, 1nM leptin 20.32 ± 2.518%, p<0.01, 10nM leptin 21.62 ± 2.363%, p<0.01, 100nM leptin 25.68 ± 2.213%, p<0.001, 1nM leptin$_{116-130}$ 27.90 ± 4.018%, p<0.001, 10nM leptin$_{116-130}$ 22.25 ± 4.954%, p<0.01, 100nM leptin$_{116-130}$ 29.81 ± 2.593%, p<0.001) but there was no difference between 0% serum

conditions. This shows that 0% serum significantly decreased cell number and no concentration of leptin was able to protect against this. However, within the 10% condition leptin$_{116\text{-}130}$ did significantly decrease cell number relative to control suggesting these concentrations were slightly toxic to cells whereas leptin at the same concentration was not.

LDH assay (n=3) showed no significant difference between conditions (p=0.1906). Further showing no protective effect of either leptin type in these conditions. Due to the differences in serum concentration 0% serum conditions could not be compared to 10% serum conditions on LDH assay. This is because serum itself produces a background reading in LDH assays, as such in direct comparison serum containing conditions will have higher LDH readings and produce a false positive on this assay (Thomas *et al.*, 2015).

It was considered if the undifferentiated HT22 cell had sufficient expression of the ObR receptor in order for leptin and leptin$_{116\text{-}130}$ to be able to enact a protective effect and further if expression changes with differentiation and therefore leptin and leptin$_{116\text{-}130}$ would have a beneficial effect on differentiated cells which could not be seen in the undifferentiated cultures. As such protein was taken from both undifferentiated and differentiated HT22 cells to run an ELISA for ObR (see **Table 3** and **Figure 25**).

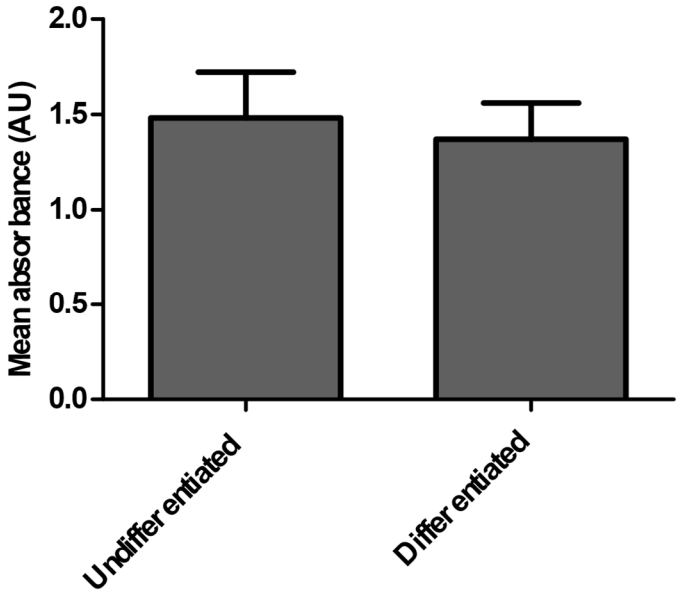

Figure 25. ObR expression undifferentiated and differentiated HT22 cells. Graph shows mean +SEM absorbance in the ELISA assay. ELISA assay demonstrated no differences in ObR expression levels in undifferentiated compared with differentiated HT22 cells ($t_{(4)}$=0.3994, p=0.71, n=5).

The ELISA assay shows no significant difference in ObR expression between undifferentiated and differentiated HT22 cells (p=0.7100; n=5). ObR expression is similar in the undifferentiated and differentiated HT22 cell line. This indicates that leptin and leptin$_{116\text{-}130}$ would be unlikely to have greater effect on the differentiated cells and as such no further exploration of the differentiated cells is warranted.

5.8 CAN A HUMAN EQUIVALENT SEQUENCE AND SMALLER FRAGMENTS OF LEPTIN$_{116\text{-}130}$ ALSO PRODUCE PROTECTIVE EFFECTS?

Given that leptin$_{116\text{-}130}$ is a murine leptin sequence and AD is a disease in humans it is important to verify that an equivalent human sequence demonstrates the same protective effects within a human cell line. Thus, the human leptin sequence leptin$_{117\text{-}125}$ was used under the conditions of the initial leptin and copper experiment (see 5.3.1) at concentrations of 0.1-10nM to compare with leptin$_{116\text{-}130}$ and full-length human leptin (Figure 26 E and F). The equivalent human leptin sequence leptin$_{117\text{-}125}$ was used as human leptin$_{116\text{-}130}$ was under patent (Grasso, Lee and Leinung, 2011) and the amino acid sequence 117-125 was suggested to be amenable to further modifications by an industrial collaborator.

Additionally, leptin$_{116-130}$ is still a large molecule to be used in an oral drug. At 14 amino acids long, it is around 1561Da as mentioned previously (see **5.1**) an ideal upper limit for an orally active drug is 500Da, as such it was investigated if yet smaller human leptin sequences could produce equivalent neuroprotective effects. The sequences chosen for this were leptin$_{116-121}$ and leptin$_{117-122}$, selected by first pepscanning the murine leptin$_{116-130}$ and identifying two neuroactive sequences, then finding the corresponding human sequences from a more limited pepscan of human leptin$_{116-130}$. These were also used at 0.1-10nM to compare with leptin$_{116-130}$ and full-length human leptin with 10µM copper chloride (**Figure 26** leptin$_{116-121}$ A and B; leptin$_{117-122}$ C and D). LDH (n=9 for all) and crystal violet (n=8 for all) assays were performed to assess viability (**Figure 26**).

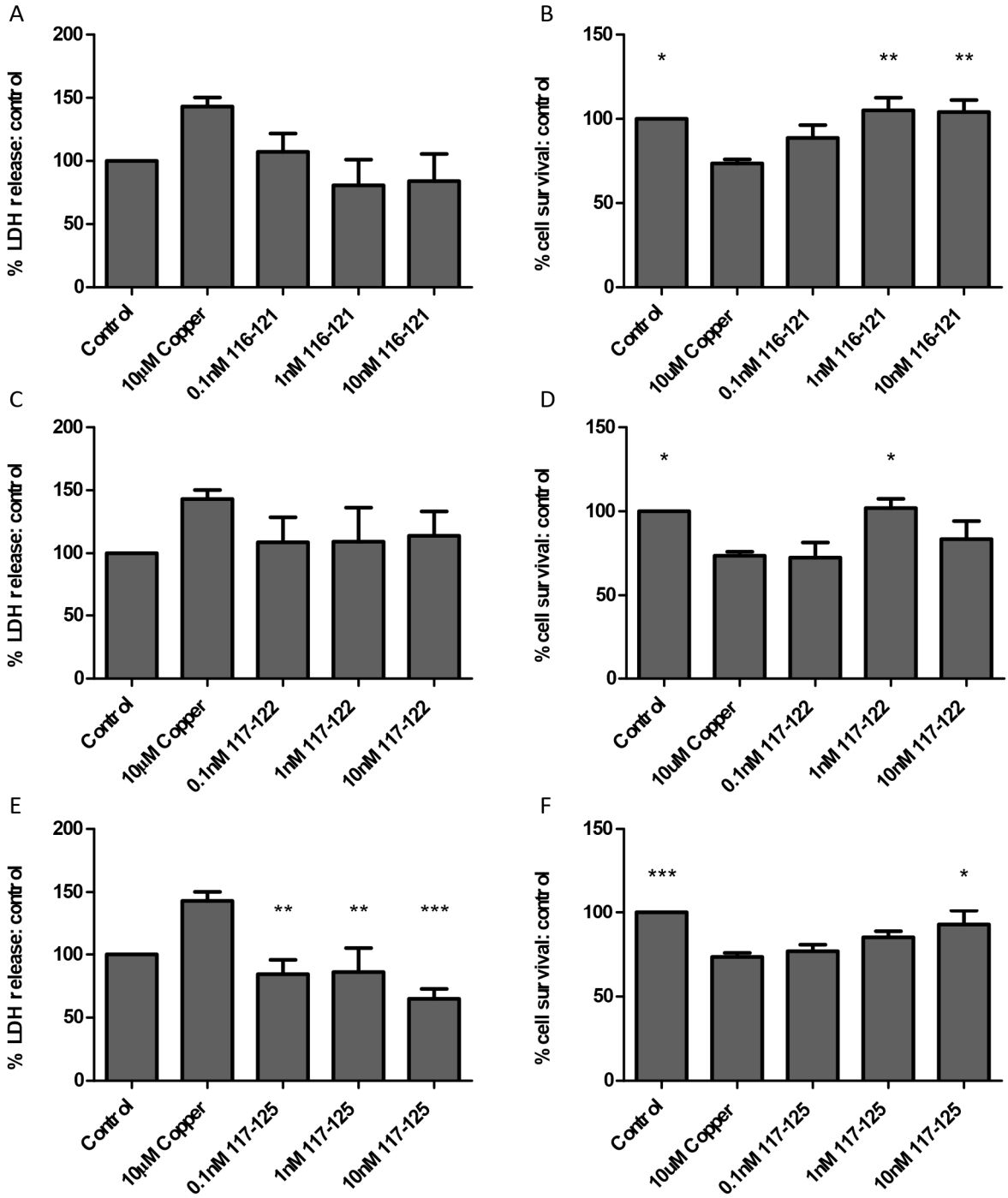

Figure 26. Leptin hexamers (116-121 or 117-122) and the human equivalent of the leptin fragment (117-125) significantly protected SHSY-5Y neurons from copper toxicity. Viability assays and photomicrographs are shown of differentiated SHSY-5Y neurons treated with copper chloride and a range of concentrations of leptin$_{116\text{-}121}$, leptin$_{117\text{-}122}$ and leptin$_{117\text{-}125}$. Bar graphs show mean +SEM % LDH release (**A**, **C** and **E**) or % cell survival (**B**, **D** and **F**) relative to control. Neither leptin hexamer demonstrated significant protective effect through LDH assay (116-121($F_{(4,39)}$=2.504, p=0.0578, n=9) **A**, 117-122 ($H_{(4)}$=7.510, p=0.1113, n=9) **C**) but 1 and 10nM leptin$_{116\text{-}121}$ and 1nM leptin$_{117\text{-}122}$ showed significantly more cell survival in the crystal violet assay (116-121 ($F_{(4,35)}$=5.202, p=0.0021, n=8) **B**, 117-122 ($F_{(4,34)}$=4.133, p=0.0078, n=8) **D**). The human sequence equivalent of leptin$_{116\text{-}130}$ here labelled as leptin$_{117\text{-}125}$ showed significantly lower LDH release than the copper condition at 0.1, 1 and 10nM

(($F_{(4,38)}$=0.4014, p=0.0005, n=9)**E**) but only 10nM showed significant differences in the crystal violet assay (($F_{(4,33)}$=5.872, p=0.0011, n=8) **F**).

The viability data for leptin$_{116-121}$ found no significant differences between the groups for LDH (p=0.0578; n=9). However, for the CV data (n=8) control (26.44 ± 0%, p<0.05), 1nM (31.45 ± 7.497%, p<0.01) and 10nM (30.55 ± 7.023%, p<0.01) leptin$_{116-121}$ had significantly increased cell number compared to copper alone.

For leptin$_{117-122}$ LDH data also showed no significant difference (p=0.1113; n=9) but for the CV (n=8) control (26.44 ± 0%, p<0.05) and 1nM leptin$_{117-122}$ (28.40 ± 5.502%, p<0.05) had significantly increased cell survival relative to copper alone.

Finally, for leptin$_{117-125}$ the LDH data (n=9) showed 0.1nM (58.66 ± 11.20%, p<0.01), 1nM (57.03 ± 19.03%, p<0.01) and 10nM (78.19 ± 7.828%, p<0.001) leptin$_{117-125}$ had significantly decreased LDH release relative to copper alone. The CV data (n=8) found control (26.44 ± 0%, p<0.001) and 10nM leptin$_{117-125}$ (19.26 ± 8.218%, p<0.05) had significantly increased cell number compared to copper.

5.9 Discussion

This chapter set out to identify if a fragment of the full-length leptin hormone could act as a neuroprotective under disease relevant scenarios in order to determine its potential as the basis of an AD therapeutic. Following these findings, I sought to assess the underlying signalling mechanisms for leptin$_{116-130}$ and compare its abilities to full-length human leptin for disease modifying effects. I assessed its role as a cognitive enhancer and its protective abilities in a hippocampal cell line. Finally, a pilot assessment of smaller fragments and a human equivalent sequence were undertaken.

In accordance with previous studies ObR was identified in the SHSY-5Y cell line both pre and post differentiation (Benomar *et al.*, 2005). However, in contrast to this study I did not find an increase in receptor expression post-differentiation, rather ObR expression remained consistent across conditions. One reason for these differences could be the differentiation method used. Whilst this previous experiment similarly used 10µM Ra to differentiate their cultures serum levels were not decreased and

cultures were maintained in this medium throughout experimentation. Triiodothyronine (T3) has been shown to greatly increase ObR in porcine hepatocyte cell cultures (Caperna *et al.*, 2005) and this hormone is found in fetal calf serum used for cell culture maintenance (Borek *et al.*, 1983). Therefore, it is possible the increased receptor expression is due to culture conditions rather than differentiation. Further, in the comparison of undifferentiated and differentiated cells this study looked at leptin binding rather than receptor expression directly.

Interestingly the ICC data for ObR in the undifferentiated cells demonstrated a pattern of localisation appearing to be nuclear. A previous study, on breast cancer, has shown similar staining patterns in cancer cells, whereby both leptin and its receptor concentrate at the nucleus and show sparse distribution in the cytoplasm (Al-Shibli *et al.*, 2017). Given that pre-differentiation, SHSY-5Y cells are a neuroblastoma cell line this lends support to this finding. This may also explain why the previous study found an increase in leptin binding to ObR post-differentiation. In the undifferentiated cells leptin would have to cross the cell membrane in order to bind to ObR at the nucleus, whereas in differentiated cells the receptor is present on the cell membrane thus making access much easier.

Once the receptor was identified I could proceed to compare the efficacy of $leptin_{116\text{-}130}$ as a neuroprotective agent in comparison to full-length leptin with both a more generalised kill condition relating to neurodegeneration and a more AD specific one. Initially MTT and LDH assays were chosen as appropriate tests of viability however as the MTT assays were being performed and analysed no consistent dose response was evident with any form of leptin used across the concentrations and the data produced a fair level of variability. In contrast, robust neuroprotection was repeatedly detected in the LDH assays. It was hypothesised that leptin may have a role in modulating mitochondrial function which renders the MTT assay inappropriate. Results from our lab indicate that leptin prevents oxidation via MAO A and B (Cheng *et al.*, no date). Other work has studied the impact of leptin on the mitochondria using the leptin deficient ob/ob mouse model. Both lean and obese ob/ob mice were

treated with saline or leptin for 5 days and the effects on their liver, extensor digitorum longus (EDL) muscle and soleus muscle studied. In saline treated obese mice mitochondria respiration was decreased in the liver and increased in EDL muscle, with no change in soleus muscle, but mitochondrial transcription factor A (TFAM; regulator of mitochondrial transcription) decreased in all areas. Protein markers for complex II, IV and ATP synthase were increased in EDL muscle of obese mice and leptin treatment had no impact on this but leptin did increase TFAM expression in liver and EDL muscle (Holmström *et al.*, 2013). In the kidneys of ob/ob mice mitochondria showed normal respiration but electron transfer was partially uncoupled from ATP synthesis (Munusamy *et al.*, 2015). This data demonstrates that leptin deficiency effects the mitochondria and does so differently in different tissues. In terms of cell culture leptin treatment of MCF-7 cells showed improved mitochondrial respiration, decreased oxidative stress and unregulated genes and proteins involved in biogenesis (Blanquer-Rosselló *et al.*, 2015). Together this shows that leptin has a regulatory role in mitochondria and as such, it is possible that an MTT assay is not appropriate to measure cell survival with leptin treatment due to the nature of the assay relying on oxidation. Taking this into consideration the LDH data leads to the conclusion that all concentrations of mouse leptin and leptin$_{116\text{-}130}$, and all concentrations bar 0.01nM human leptin were able to protect from copper chloride induced cytotoxicity. Further there were no significant differences between any of the leptin concentrations of any species used under toxic conditions indicating leptin$_{116\text{-}130}$ is equally effective as full-length leptin in its protective properties. This demonstrates that mouse leptin can equally protect the human SHSY-5Y cells from toxic insult as human leptin so any differences in the fragment's abilities should not be impeded by it being a mouse sequence. Further it shows leptin$_{116\text{-}130}$ has equal abilities as full-length leptin as a neuroprotective.

Following this result the ability of leptin$_{116\text{-}130}$ and human leptin to protect against an AD-specific toxic state was explored. This experiment was streamlined using only three leptin concentrations and given that leptin$_{116\text{-}130}$ had shown comparable effects to both human and mouse leptin in the prior experiment it was decided to continue forward only comparing to human leptin. Given the discussed issues with the MTT assay the CV assay was chosen to replace it as a marker of cell viability. Combined

these assays demonstrated both leptin and leptin$_{116-130}$ treated conditions maintained higher cell number and produced less LDH in comparison to Aβ treated conditions. This shows that more cells remained attached to the culture plate substrate, and therefore are healthy, and there was reduced membrane rupture and therefore less necrosis or late stage apoptosis when cells were treated with leptin or leptin$_{116-130}$ in the presence of Aβ. These results agree with previous studies that show leptin is able to protect neurons from toxic insults (Weng *et al.*, 2007; Doherty, Oldreive and Harvey, 2008; Zhang and Chen, 2008; Doherty *et al.*, 2013; Davis, Mudd and Hawkins, 2014) and additionally just a fragment of this molecule in the form of leptin$_{116-130}$ can replicate its protective effects. Further to this research leptin$_{116-130}$ has been shown to facilitate synaptic plasticity, regulate synaptic transmission, increase GluA1 surface expression and AMPA receptor insertion in the synapse in hippocampal neurons, as well as overcome Aβ-induced LTP inhibition and LTD induction, in a manner that mirrors the effects of leptin itself (Malekizadeh *et al.*, 2017).

Leptin has been shown to mediate its neuroprotective effects via PI3K (Doherty, Oldreive and Harvey, 2008; Doherty *et al.*, 2013; Wen *et al.*, 2015; Gavello, Carbone and Carabelli, 2016) and STAT3 (Doherty, Oldreive and Harvey, 2008; Guo *et al.*, 2008) signalling cascades. As such it was explored whether application of leptin$_{116-130}$ increased phosphorylation, and hence activation, of STAT3 and Akt, a downstream signalling molecule in the pro-survival PI3K pathway. Leptin$_{116-130}$ significantly upregulated these pathways in SHSY-5Y cells indicating it is acting in the same manner as leptin to produce its protective effects. This data is further supported by research showing selective inhibition of these pathways eliminates the protective effects of leptin$_{116-130}$ (Malekizadeh *et al.*, 2017). Work has suggested that leptin$_{116-130}$ does not bind to ObRb. GT1-7 cells transfected with ObRb were shown to have increased reporter gene activity in response to mouse leptin but not leptin$_{116-130}$, further COS-7 cells transfected with ObRa show high binding with mouse leptin, but binding was no different than control with leptin$_{116-130}$. Using db/db mice deficient in ObRb, leptin$_{116-130}$ treatment significantly reduced blood glucose and prevented weight gain, despite no apparent change in food intake (Grasso

et al., 1999). Taken together with the signalling data this suggests that leptin$_{116\text{-}130}$ may be able to activate leptin signalling pathways and enact leptins effects without using ObR.

Within AD key molecules have been highlighted in the involvement of disease progression as such it is important to understand if any potential treatment can modulate these molecules within the disease paradigm. Following a subtoxic dose of amyloid beta alongside protective concentrations of leptin and leptin$_{116\text{-}130}$ protein extracts from cultures were assessed for changes in these AD biomarkers. However, no significant changes in biomarker levels were detected, with either leptin or with amyloid beta itself. This suggests a flaw in the initial experimental design. Of 8 experimental repeats only 3 were able to reliably demonstrate an increase in tau phosphorylation which was set as the criteria for use within the experiment. It is suggested that the incubation time with amyloid beta is too short to reliably generate changes in the markers chosen. Although other experiments have used this concentration of Aβ over much shorter time periods (0-24h) they were either using a cell line much more vulnerable to Aβ toxicity e.g. teracarcinoma cells which show significantly reduced viability after 24h with Aβ at this concentration (Costa *et al.*, 2012), or were looking for acute responses e.g. initial calcium response to Aβ in PC12 cells (Gilson *et al.*, 2015), whereas I aimed to study much slower cumulative effects and demonstrated that Aβ did not induce cell death in these cultures after 96h. Experiments looking at primary cultures found conflicting effects. 48h treatment of primary mouse cortical neurons with 1μM Aβ found increases in both p-tau and endophilin 1 (Doherty *et al.*, 2013) whereas treatment of rat hippocampal neurons saw no effects of Aβ until the cells were aged (Calvo-Rodríguez *et al.*, 2017). In an experiment where Aβ was given to an animal prior to tissue extraction the treatment period was 10 days after which cognitive impairment was seen and increased SOD and ROS detected in hippocampal slices (Calvo-Rodríguez *et al.*, 2017). These results confirm that length of treatment time may be essential in elucidating changes in these biomarkers.

Despite this the trends in the data may be worth assessment. The ratio of phosphorylated tau to tau ELISA results suggest that Aβ has increased tau phosphorylation at serine 262 as has been described previously (Manassero *et al.*, 2016; Bennett *et al.*, 2017). Leptin and leptin$_{116\text{-}130}$ had very little impact on the ratio of p-tau: tau, though leptin$_{116\text{-}130}$ might appear to have been able to prevent tau phosphorylation at ser262 a t-test on 1μM Aβ cultures compared to Aβ with leptin$_{116\text{-}130}$ treatment renders this unlikely (p=0.2342). Previous, studies have suggested that leptin prevents tau protein phosphorylation and does so by stimulation of the AMP-activated protein kinase (AMPK) pathway (Greco *et al.*, 2009; Li *et al.*, 2016). However, this result requires further investigation as a more recent study has shown that activation of AMPK increased tau phosphorylation at multiple sites including ser262 (Domise *et al.*, 2016). Leptin-resistant mice have been shown to display elevated levels of tau phosphorylation and cells cultured with leptin have reduced p-tau (Platt *et al.*, 2016). Although this mouse model study did not look at the same phosphorylation site as the current study, research from OLETF rats which have impaired leptin signalling do show increased p-tau at Ser262 but only from 42 weeks old (Jung *et al.*, 2013). This may be an indicator that culture treatment length needed to be increased or that cultures were too young to see significant changes.

The ABAD ELISA demonstrates no obvious changes in levels across any treatment conditions. ABAD is upregulated in neurons affected by AD and it is through interaction with this molecule that Aβ causes mitochondrial dysfunction in the disease (Lustbader *et al.*, 2004). As leptin appears to have a role in the mitochondria I aimed to identify if leptin could modulate ABAD. However, no connection was seen and as yet little is published on the roles of leptin in this area.

Oxidative stress has been shown to increase β-secretase (β-site amyloid precursor protein cleaving enzyme 1: BACE1) which is key in the production of Aβ. In turn Aβ increases OS and hence in a positive feedback loop leads to an increase in BACE1 and its own production (Tamagno *et al.*, 2007; Zhao *et al.*, 2007; Chakrabarty *et al.*, 2010). Further, OS has been linked with increased production of APP (Picone *et al.*, 2015) and nuclear factor-κB (NF-κB) has been seen to increase APP expression at

supraphysiological levels of Aβ (Chami *et al.*, 2012). As such it is expected that addition of Aβ would increase APP levels in these cultures. However very little change was seen in APP levels across conditions and leptin but not $leptin_{116-130}$ seemed likely to have affected this (t-test Aβ compared to leptin with Aβ p=0.1074; Aβ compared to $leptin_{116-130}$ with Aβ p=0.3342). Leptin has been shown to attenuate NF-κB (Marwarha *et al.*, 2014) which suggests it can indirectly modulate APP. However, this data suggests the effect is independent of amino acids 116-130.

Endophilin 1 is expressed only in the brain and localises at presynaptic nerve endings, whereas endophilins 2 and 3 are found in peripheral tissues. It has been identified as being necessary for endocytosis and synaptic vesicle formation (Reutens and Glenn Begley, 2002). It has also been shown to have a role in the BBB as knocking down endophilin 1 in epithelial cells has been shown to decrease BBB permeability (Liu *et al.*, 2014). BBB disruption occurs in AD and has been suggested to precede Aβ plaque formation (Erickson and Banks, 2013; Festoff *et al.*, 2016). Treating a BBB cellular model with Aβ has shown to increase endophilin 1 levels and increase its permeability (Liu *et al.*, 2016). Further, endophilin 1 levels have been shown to be increased in AD affected neurons and increasing endophilin 1 levels in Aβ rich environments disrupts LTP and leads to synaptic loss and dysfunction (Yu *et al.*, 2018). The results from the current experiment agree with this as they indicate that endophilin 1 was increased with Aβ. However, levels were also increased with both leptin and $leptin_{116-130}$ and co-treatment of either leptin with Aβ produced similar levels of endophilin 1 increase. A previous study has shown leptin was able to prevent Aβ-mediated upregulation of endophilin 1 in vitro (Doherty *et al.*, 2013), although this study used 100nM leptin compared to the 1nM leptin used here. However, leptin insensitive Zucker fa/fa rats also demonstrate increase endophilin 1 levels (Doherty *et al.*, 2013) as such it is unexpected that leptin treatment alone would increase endophilin 1 levels in healthy cells as seen here. An explanation for this could be found in the signalling pathways. I have shown that leptin and $leptin_{116-130}$ increase activation of the PI3K pathway. This pathway has also been implicated in the production of PI(3,4)P2 which mediates fast endophilin-mediated endocytosis (Hawkins and Stephens, 2016). Further leptin has also been shown to have different functioning in cells that are firing and at rest, with 1nM

leptin causing a slow membrane hyperpolarisation in mouse chromaffin cells at rest but preserved action potential firing and increased catecholamine secretion during stimulation (Gavello *et al.*, 2015) and to increase vesicle trafficking and secretion of catecholamines in PC12 cells (Than *et al.*, 2011). Together, this research indicates a more complex relationship between leptin and endophilin than has previously been seen and may hint at a dose and state dependent role in vesicle release.

Within many forms of AD, mutations in the presenilin 1 gene have been linked with both pathologic and behavioural symptoms (Larner, 2013) including the production of neurotoxic Aβ. Presenilin 1 forms part of the γ-secretase enzyme complex which makes the secondary cut in APP to form Aβ. Changes in the presenilin 1 component (e.g. prevention of phosphorylation or mutation of ser367) lead to increases in Aβ production (Bustos *et al.*, 2017). Further to earlier mentioned research that demonstrates OS increases BACE 1 it has been shown that this mechanism is via γ-secretase and the JNK pathway, whereby OS increases JNK activation which mediates increased expression of presenilin 1 (and hence γ-secretase) and BACE 1, these in turn increase the production of Aβ which induces greater OS (Tamagno *et al.*, 2008). My results do not suggest that amyloid beta increased presenilin 1. Whilst leptin and $leptin_{116\text{-}130}$ treatments in the presence of Aβ appear to have decreased presenilin 1, t-tests between Aβ treatment and these conditions suggest neither leptin nor $leptin_{116\text{-}130}$ were likely to have impacted presenilin levels (leptin $p=0.475$, $leptin_{116\text{-}130}$ $p=0.2447$). A previous study which found that plasma leptin levels negatively correlate with brain presenilin 1 levels and leptin treatment decreased expression of presenilin 1 in H4 and SHSY-5Y cells (Niedowicz *et al.*, 2013). However, this experiment used a three times higher leptin concentration and Aβ was seen to increase γ-secretase. As presenilin 1 was not increased in our cultures this may have prevented leptin inducing an effect.

Whilst the subtoxic dose of amyloid beta was not sufficient in this time frame to reliably increase p-tau levels and generate significant differences in the ELISA data some interesting trends are elucidated. In my cultures, leptin and $leptin_{116\text{-}130}$ largely demonstrated the same trends in each

biomarker, further demonstrating they have similar actions. Little changes were seen in p-tau, ABAD or presenilin 1 and endophilin 1 was increased with both leptin and leptin$_{116-130}$ treatments alone. This is contrary to previous research and suggests that the relationship between leptin concentration and vesicle release might be vital in understanding these discrepancies. The APP results may indicate a trend which requires further investigation as leptin but not leptin$_{116-130}$ may be able to decrease the source of Aβ (APP) under AD conditions. This would suggest a difference in action between leptin and leptin$_{116-130}$ which might be important for its disease-modifying effects. Overall, these results must be treated with caution as I am only able to make suggestions based on trends in the data rather than strong conclusions from significant differences.

Whilst unable to provide firm conclusions on leptin and leptin$_{116-130}$ providing modifications to disease biomarkers in AD another important factor in the disease is the loss of episodic memory. As I have shown leptin$_{1160-130}$ has neuroprotective effects and further work demonstrates its ability to enhance hippocampal synaptic plasticity (Malekizadeh *et al.*, 2017) the implications of its ability to treat memory dysfunction, and hence protect against neurodegeneration in the early stages of AD, become an interesting line of enquiry. As such mice were tested on the episodic-like OPC memory task to study the acute effects of both leptin and leptin$_{116-130}$ on this type of memory. In pre-treatment testing of the component parts of the OPC task no significant differences were found between groups of mice although on the OP task, mice in the pre-treatment saline group, were not found to perform above chance in discriminating between novel and familiar objects when location of the object was the determining factor. Upon further analysis of the data from individual test days a preference for the object on the left in the second day which negatively discriminated against animals in the saline group was identified. Further removal of animals who showed lack of exploration (less than five seconds) in the encoding phases lead to above chance discrimination index scores. Therefore the combination of these factors is believed to be the source of this difference.

The 3-minute data demonstrates all groups performed significantly above chance with no difference between the saline, leptin and leptin$_{116\text{-}130}$ group, however there was a trend for the saline group scores to a lower discrimination index than the leptin or leptin$_{116\text{-}130}$ groups. Previous research suggests that performance on this task significantly declines after the first minute of a trial, as the novel object is more interesting initially but preference for it steadily declines over the test phase (Clark, Zola and Squire, 2000). As such data was also analysed up to the 1-minute time point and both leptin and leptin$_{116\text{-}130}$ showed significantly better performance than the saline control group demonstrating powerful cognitive enhancing effects. This shows that not only can leptin enhance the specific type of memory that declines in AD but that just a fragment of this molecule is necessary for this action. Leptin has been shown to protect against Aβ-induced impairments in spatial memory (Tong *et al.*, 2015) so it is likely both leptin and leptin$_{116\text{-}130}$ can protect against the impact of Aβ on hippocampal-dependent memory.

It is important to note that leptin has previously been seen to have anxiolytic effects (Liu *et al.*, 2010; W. Wang *et al.*, 2015; Tyree, Munn and McNaughton, 2016) which could impact my results as less anxious animals are likely to explore more freely. However, as all treatment groups explored equally in both sample and test phases during the OPC, this was not seen to be the case. Given the extensive handling and habituation of animals prior to experimentation this result is unsurprising as anxiety levels would have already been very low.

Another side effect of leptin is weight loss. As its originally identified function was as a satiety hormone it was important to monitor the animals' weights over the course of injections. No significant changes were seen in weight across the four days in any group or between any treatment groups, so neither leptin nor leptin$_{116\text{-}130}$ treated animals showed any weight loss compared to the saline controls which given the short time span of this experiment is perhaps not surprising. Other studies have found weight loss in leptin treated animals within a day (Peters, Simasko and Ritter, 2007) although they used prolonged continuous leptin exposure rather than acute daily injections as in my experiment. To assess

if any longer-term changes occurred in the mice, brains were extracted 72h after final treatment and assessed for ObR and total leptin levels. No changes in ObR expression were seen in the leptin treated mice and no changes in brain leptin levels were seen. Previous research has found that obese patients with type 2 diabetes had decreased leptin receptor and increased leptin plasma concentrations. Following treatment normalisation of receptor levels occurred but leptin levels did not change (Olczyk *et al.*, 2017) Further, in an experiment looking at the effects of obesity on placenta and foetus, increasing leptin levels reduced placental and foetal liver expression of ObR (Mazzucco *et al.*, 2013). However, in the brains of mice a study found fasting increased ObR in anterior pituitary but decreased expression in hypothalamus (Cai and Hyde, 1999). Together these suggest that the relationship between leptin and ObR expression is not clear cut and changes may occur differently in different areas of the body and brain, this may account for the variability in the receptor expression data. In terms of leptin levels within the brains itself, research suggests the half-life of circulating leptin is 40.2 minutes (Burnett *et al.*, 2017) as such it may be expected that after 72h no determinable differences could be found.

Given the variation in the leptin level data it was thought leptin levels in the brains of these animals might correlate with discrimination index scores. As leptin and $leptin_{116\text{-}130}$ was able to improve episodic-like memory perhaps those mice who had higher brain leptin levels were those who had performed better on the task. No significant correlation was found between brain leptin levels and discrimination index. All mice combined, saline and $leptin_{116\text{-}130}$ treated mice showed weak positive correlations, but leptin-treated mice showed a weak negative correlation. Interestingly the strongest correlation was seen in the $leptin_{116\text{-}130}$ group although the R^2 value was still quite low. Whilst little work has been done on natural healthy variances in leptin and cognition levels *per se*, the combined understanding that leptin deficiency lessens memory performance and increasing leptin improves memory performance suggests that to a degree higher brain leptin levels could evoke better memory performance within healthy individuals.

As leptin and leptin$_{116-130}$ were neuroprotective in a human cell line and increase cognitive performance in a murine model it was predicted that both could also be neuroprotective in HT22 cells, a mouse hippocampal cell line. As this cell line was a new model to our lab group initially pilot experiments into appropriate methods of inducing cell death were investigated. Previous studies have induced cell death by 0.5mM – 5mM glutamate treatment, which inhibits cystine uptake via the X_c cystine/glutamate antiporter system leading to reduced glutathione and death (Rössler *et al.*, 2004; Kulawiak and Szewczyk, 2012; Lee *et al.*, 2015), and by serum withdrawal (Rössler *et al.*, 2004). Results demonstrated that neither 5mM glutamate nor reduction of serum to 1% for 24h could significantly reduce cell numbers relative to controls but complete removal of serum from the medium could. As such this was chosen as the model to investigate the protective properties of leptin and leptin$_{116-130.}$ Cells were treated with a range of leptin concentrations both with and without serum and LDH and CV assays were performed. The CV assay showed all conditions with no serum had significantly lower cell numbers after 24h compared to serum containing control. No condition containing serum and any concentration of leptin showed significant changes in cell number relative to the 10% serum containing control, indicating leptin itself was not toxic to these cells. However, all leptin$_{116-130}$ conditions showed small but significant reductions in cell number compared to the 10% serum control showing these concentrations were slightly toxic to cells. This may indicate a more potent action of leptin$_{116-130}$ than leptin in this cell line. No leptin or leptin$_{116-130}$ condition in the 0% serum experimental condition had higher cell number than the 0% control, though, indicating that neither leptin type could rescue cell number from this kill condition. These results were supported by the LDH assay where no difference was found between any concentration of either leptin in the 0% condition relative to the 0% serum control. These results show neither leptin nor leptin$_{116-130}$ were able to protect HT22 cells from complete serum withdrawal induced cell death. One explanation for this result considered was that in these undifferentiated cells ObR expression may not have been sufficient for leptin to have an effect and with differentiation this may change. An ELISA comparing ObR expression in undifferentiated and

differentiated HT22 cells found no difference in expression. Very little research has been done on leptin in HT22 cells, however one study has shown leptin was able to protect against glutamate-induced cytotoxicity by counteracting glutamate-induced zinc-dyshomeostasis and mitochondrial activation (Jin, Ni and Li, 2018). As this study was also performed on undifferentiated HT22 cells it supports the idea that leptin is able to act on these cells. Very little research has also been done on the serum withdrawal kill condition used in this experiment. Previous research has found compounds that could protect from glutamate could not protect from 24h serum withdrawal (Rössler *et al.*, 2004), have used serum withdrawal over much shorter time periods and found protection (2h (Olianas, Dedoni and Onali, 2017)) or used pre-treatment with their compound of interest before serum withdrawal (Cesarini *et al.*, 2018). It is possible that the method requires further refinement or that it is using cell death pathways leptin cannot rescue from. Research suggests serum deprivation in HT22 cells is induced by a caspase-independent apoptosis by upregulation of apoptosis-inducing factor (AIF) (Steiger-Barraissoul and Rami, 2009). In hypothalamic proopiomelanocortin-expressing neurons AIF deficiency results in improved leptin sensitivity (Timper *et al.*, 2018) but leptin upregulates AIF in rat sperm (Almabhouh *et al.*, 2017). This indicates a complex cell type-dependent relationship between leptin and apoptosis, hence further research into how leptin interacts with undifferentiated HT22 cells and if this relationship changes with differentiation should be explored.

The final questions this chapter aimed to explore were whether a human sequence equivalent of the murine leptin$_{116\text{-}130}$ would have the same neuroprotective powers, and further if even smaller leptin fragments, leptin$_{116\text{-}121}$ and leptin$_{117\text{-}122}$, could also generate these effects. Whilst none of the LDH assays demonstrated a significant increase in membrane rupture in the copper condition compared to control, the CV assays and the LDH assays with leptin$_{117\text{-}125}$ were able to show protection. As such trends in the LDH data and the significance in the CV data will be discussed.

Human leptin$_{117\text{-}125}$ all concentrations were shown to have significantly lower LDH levels than copper chloride treatment group. In the CV assay only 10nM leptin$_{117\text{-}125}$ demonstrated a significant protective effect. As both leptin$_{116\text{-}130}$ and full-length leptin were protective at 1nM this suggests the abilities of leptin$_{117\text{-}125}$ may not be as potent. However, I would suggest that the human sequence leptin$_{117\text{-}125}$ can produce equivalent effects to full-length human leptin and murine sequence leptin$_{116\text{-}130}$ and further viability analysis would support this.

For leptin$_{116\text{-}121}$ all concentrations seem to have comparable LDH levels to control and significantly higher cell number is seen in the CV assay relative to the copper condition at 10 and 1nM. This is comparable to the abilities of leptin$_{116\text{-}130}$ and full-length leptin. This compared with leptin$_{117\text{-}125}$ may suggest the amino acid at 116 in this sequence is important for action at lower concentrations.

Leptin$_{117\text{-}122}$ produced less obvious trends. In the LDH assay this leptin produced very variable results though means are very similar to control which may suggest some protective ability. In the CV assay 1nM was able to protect cells but 0.1 and 10nM treatments were very similar to copper treated cultures. This suggest some concentration dependent protective abilities but investigation into leptin$_{117\text{-}122}$'s actions are required to understand this finding.

Altogether, these results may indicate that an even smaller neuroactive peptide sequence can be a promising therapeutic target and that further investigations should be carried out with leptin$_{116\text{-}121}$ not leptin$_{117\text{-}122}$ for the purpose of AD treatment.

Overall the results from this chapter have shown that the fragment leptin$_{116\text{-}130}$ demonstrates neuroprotective effects that mirror the abilities of full-length human leptin both in action and signalling pathways. Further, it has been demonstrated that both leptin and leptin$_{116\text{-}130}$ can enhance cognitive performance on a disease-relevant memory task and in combination with other research shows its important role in improving hippocampal-related memory. Further it is possible that an even smaller 6

amino acid human sequence of leptin in the form of leptin$_{116\text{-}121}$ can also produce these neuroprotective effects and as such is a valid target for therapeutic development.

6 CHOLECYSTOKININ 1 RECEPTOR AGONISM PROTECTS A NEURAL CELL LINE FROM INDUCED CELL DEATH

6.1 INTRODUCTION

Although CCK signalling via the CCK1R was initially labelled as having purely satiety-related functions exploration of this receptor has indicated receptor expression within the brain, a role in memory and in cell survival. Additionally, differential sensitivity to the CCK hormone with age suggests a pivotal relationship between CCK, ageing, memory and neuroprotection which remains to be explored. As such CCK may have an important role in age-related diseases, such as Alzheimer's, and hence manipulation of its signalling has potential as a therapeutic. One difficulty in determining this from previous studies is that CCK1R and CCK2R are often activated by the same forms of CCK, though to different degrees. As CCK-8 is the most abundant form in the brain, this is often used as the receptor agonist in the study of CCK's role in memory. It has been shown that treating rats with CCK-8 improves performance on the Morris water maze test, suggesting either improved retention of platform location or higher resistance to memory extinction (Voits *et al.*, 2001; Sadeghi, Reisi and Radahmadi, 2017). However, this form of CCK activates both CCK1R and CCK2R therefore making it impossible to identify which receptor is responsible for these effects or if in fact both are required. Given the evidence from OLETF rats lacking CCK1R demonstrating memory impairments in the same test, it could be suggested CCK1R activation is sufficient to produce these memory enhancing effects. As such, to explore CCK1R activation as a potential therapeutic target in its own right, a specific receptor agonist is needed.

A71623 and A70874 were two CCK-4 based peptides explored for their selectivity as CCK receptor agonists. It was shown that both acted as agonists in the ileum and pancreatic amylase release and their effects were blocked by selective CCK1R antagonist L-364,718. However, A71623 was a full agonist and A70874 only partial agonist of phosphoinositide breakdown in the pancreas (Lin *et al.*, 1991). Further exploration of A71623 found it to be potent in the suppression of food intake in CD1 mice and cynomolgus monkeys, and to a lesser degree in beagles, with these effects blocked by specific

CCK1R antagonist A70104 (Asin, Bednarz, Nikkel, Gore and Nadzan, 1992). In comparison to CCK-8 it was shown to have greater potency and duration of action in rats and at doses exceeding those needed to suppress food intake A71623 also reduced spontaneous locomotor activity (Asin, Bednarz, Nikkel, Gore, Montana, *et al.*, 1992). This evidence suggests A71623 is a potent and selective agonist of the CCK1R through which I could explore the roles of this receptor.

This chapter aims to identify whether CCK1R activation can prevent cell death using the SHSY-5Y human neuroblastoma model. Initially work was carried out on the undifferentiated cell line to establish quickly if CCK1R agonism by A71623 could protect against apoptosis and necrosis using LDH and MTT assays to measure cell viability. Following this, the ability of A71623 to modulate nitrosative and oxidative stress, both of which are implicated in ageing and neurodegeneration, was determined using cell fluorescent and biochemical techniques. After establishing A71623's abilities to protect against cell death in undifferentiated SHSY-5Y cells, investigation of the fully differentiated model was warranted. The neuroprotective abilities of CCK1R agonism through A71623 were tested using neurodegenerative- and AD-relevant toxic conditions and the signalling mechanisms behind this ability were assessed. Since CCK1R deficient rodents exhibit upregulation of biomarkers of ageing and neurodegeneration (Jung *et al.*, 2013) biochemical techniques were used to establish modulation of a range of these biomarkers by A71623 in AD-relevant conditions. Finally, it was investigated if agonism of this receptor may play a role in murine memory through an episodic-like memory paradigm.

6.2 THE RECEPTOR CCK1R IS EXPRESSED IN UNDIFFERENTIATED SHSY-5Y CELLS

Whilst CCK receptor expression has been seen in differentiated SHSY-5Y cells (Wen *et al.*, 2012), our initial studies used the undifferentiated cell line. To ascertain the presence of the cholecystokinin receptor of interest, CCK1R, in our undifferentiated model immunocytochemistry (**Figure 27**A) and Western blot (**Figure 27**B) were used (see **Table 3** for antibody concentrations).

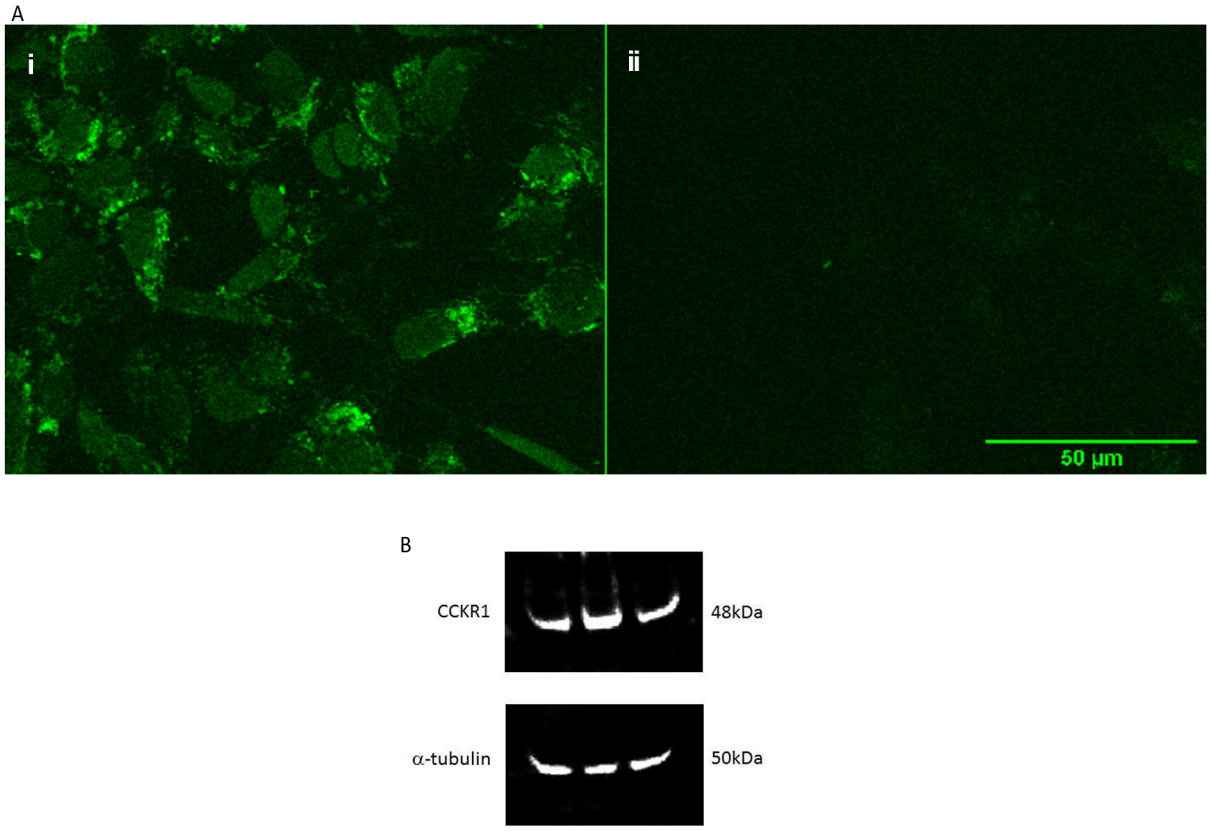

Figure 27. The CCK1R is expressed in undifferentiated SHSY-5Y cells. A – Fluorescent images of undifferentiated SHSY-5Y cells are shown labelled with CCK1R primary antibody and fluorescein-conjugated secondary (i) and labelled with secondary in the absence of the primary antibody (ii). Images were taken using 63x magnification. Greater staining in the primary antibody-containing condition indicates the presence of the receptor in this cell line. B – CCK1R expression in the undifferentiated SHSY-5Y cell line as detected in protein extracts by western blot and α-tubulin loading control for the same protein samples.

The ICC demonstrates green staining in the primary containing condition indicating that the undifferentiated SHSY-5Y cells do express the CCK1R (n=6). This is confirmed by the detection of bands in the western blot where protein from three samples demonstrates CCK1R expression with little variation in expression levels as demonstrated by similar band size and equal protein loading (n=3).

6.3 CCK1R AGONISM BY A71623 CAN PROTECT AGAINST CELL DEATH IN VITRO

As inhibition of the CCK1R has been linked with apoptosis (Carrillo *et al.*, 2009) and application of CCK can rescue β-cell cultures from cytokine induced cell death (Lavine *et al.*, 2010), this study aimed to identify if agonism of the CCK1R, through application of A71623, alone could protect cells from induced cell death. As such undifferentiated SHSY-5Y cells were treated with A71623 in a range of

concentrations from 2-50nM, selected based on previous research (Lin *et al.*, 1991). To test the protective ability of this compound cells underwent combined serum and glucose deprivation (SGD) or heatshock to induce cell death.

6.3.1 CCK1R agonism protects against apoptosis induced via combined serum and glucose deprivation.

Combined serum and glucose deprivation (SGD) is used to induce apoptosis in cellular models and reflects the molecular mechanisms of ischemia-induced brain injury in neurons (Mousavi *et al.*, 2016). Ischemia is a risk factor for AD and is thought to increase amyloidogenic processing of APP (Salminen, Kauppinen and Kaarniranta, 2017), as such this is a relevant model through which to test protective abilities of a new treatment. Therefore, undifferentiated SHSY-5Y cells were deprived of serum and glucose and treated with 2, 10 or 50nM of the CCK1R agonist A71623 for 96h before MTT and LDH assays were used to asses cell viability (**Figure 28**A and B). Following this, representative photomicrographs were taken to visually demonstrate differences between SGD alone and SGD with A71623 treated conditions (**Figure 28**C).

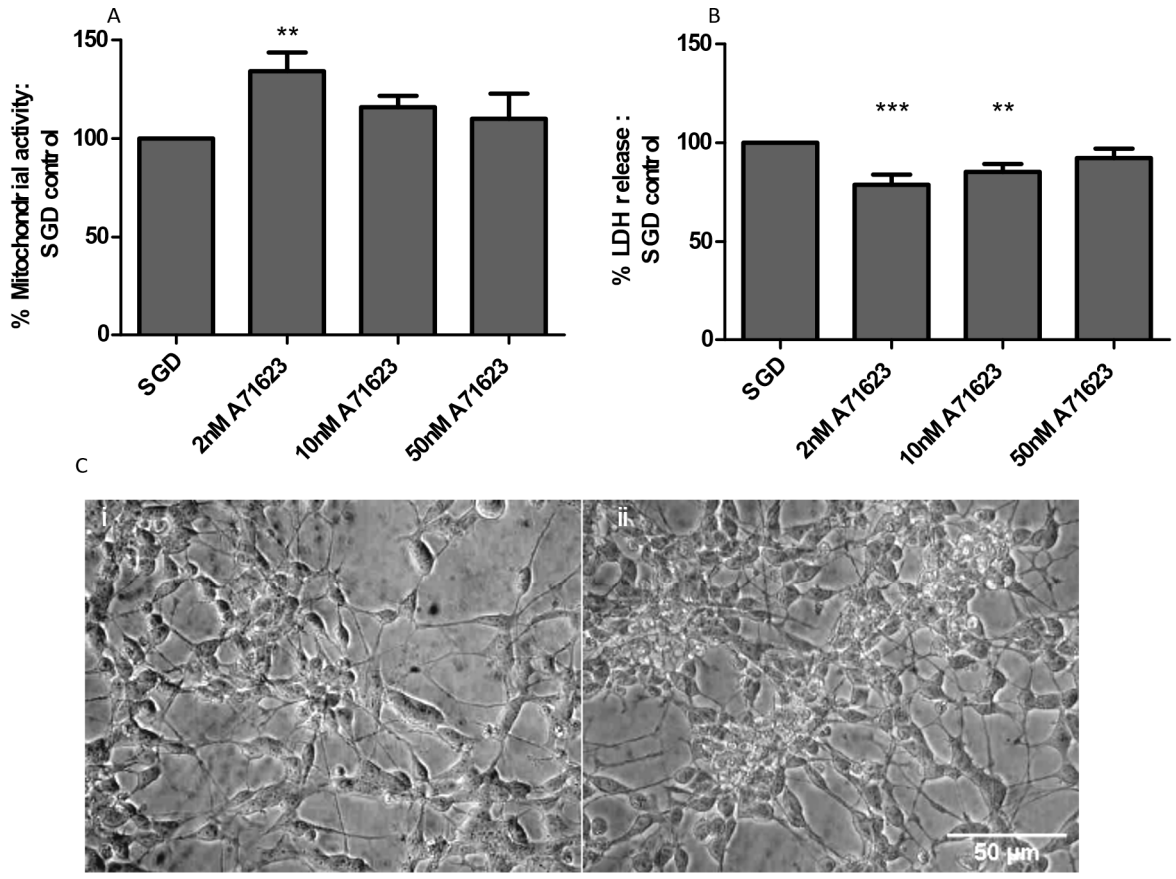

Figure 28. CCK1R agonism by A71623 can protect against SGD-induced cell death. Graphs show mean + SEM. Significance relative to SGD control is denoted by asterisks. A- Shows improved mitochondrial activity with 2nM A71623 application as measured by the MTT assay ($H_{(3)}$=9.390, p=0.0245, n=7) . B – shows LDH release measured from cultures with both 2 and 10nM A71623-treatments demonstrating lower LDH release relative to SGD condition alone ($H_{(3)}$=22.66, p<0.0001, n=12). C – Representative photomicrographs of cells (x63 magnification) in SGD (i) and 2nM A71623 (ii) treated conditions.

The results of the MTT assay (n=7) showed only 2nM A71623 had significantly increased mitochondrial activity by 34.2 ± 9.413% compared to SGD alone (p<0.01). The LDH data showed both 2nM A71623 (p<0.001) and 10nM A71623 (p<0.01) had significantly decreased LDH release (25.50 ± 5.076% and 20.33± 4.016%, respectively) compared to SGD alone. Photomicrographs demonstrate higher cell number in the 2nM A71623 treated condition, visually, compared to SGD alone. Together these results demonstrate that cells under SGD conditions when treated with 2nM A71623 had better mitochondrial health and less membrane rupture. Combined with the results of the photomicrograph this demonstrates this concentration of A71623 is successfully protective against SGD-induced cell death.

6.3.2 CCK1 agonism can protect cells from necrosis induced via heatshock

Heatshock induces a rapid stress response (heatshock response; HSR) and necrosis within cell cultures. This HSR can also be induced by other factors such as heavy metals, toxins and infections and leads to the induction of heatshock protein (HSP) genes, regulated by heatshock transcription factor 1 (HSF1) (Mahat and Lis, 2017). HSF1 and HSPs have been linked with various diseases, for example, HSF1 degradation increases α-synucleinopathy (Kim *et al.*, 2016), inhibition of HSP90 rescues synaptic function in an AD mouse model (B. Wang *et al.*, 2017) and HSP90 regulates tumour invasiveness (Wong and Jay, 2016). Hence heatshock is a relevant model through which to explore new protectives. As such, undifferentiated SHSY-5Y cells were heatshocked at 60°C for 2 minutes then treated with 2, 10 or 50nM A71623 after cooling. After 24h MTT and LDH assays were performed to measure cell viability and representative photomicrographs taken to visually demonstrate differences between heat-shock alone and heatshock with A71623 treatment conditions (**Figure 29**).

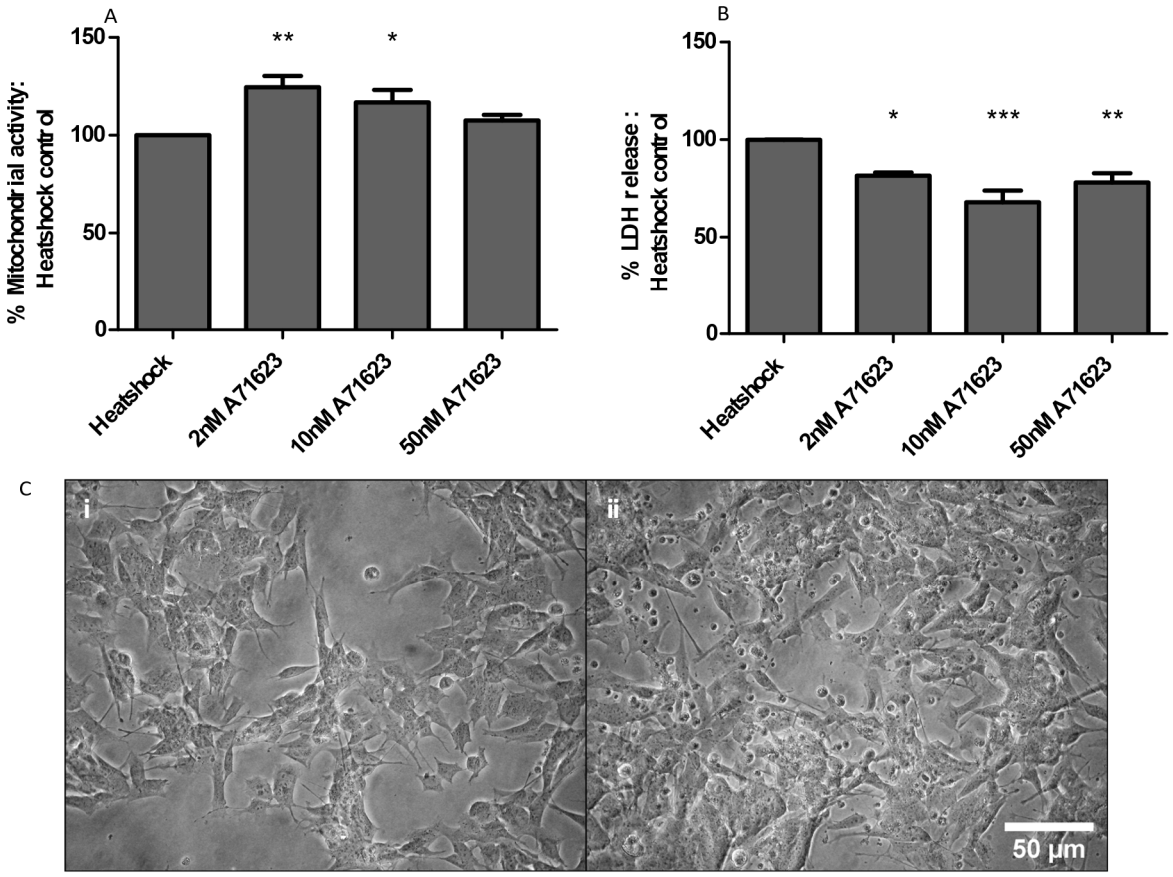

Figure 29. CCK1R agonism protects against heatshock in undifferentiated SHSY-5Y cells. Graphs show mean +SEM. Significance relative to heatshock control denoted by asterisks. A – shows improved mitochondrial activity after treatment with 2nM and 10nM A71623, as detected by MTT assay ($H_{(3)}$=13.33, p=0.004, n=5). B – The graph shows all concentrations of A71623 used (2, 10 and 50nM) caused less LDH detection in media as found by LDH assay ($F_{(3,16)}$=11.54, p=0.003, n=5). C - Phase contrast photomicrographs display either cells in the heatshock alone (i) or with 2nM A71623 (ii) under 63x magnification.

The MTT data demonstrated that 2nM ($p<0.01$) and 10nM A71623 ($p<0.05$) significantly increased mitochondrial activity compared to SGD alone (24.6 ± 5.675% and 116.8 ± 6.443%, respectively). Whereas the LDH assay showed all concentrations of A71623 decreased LDH release in cultures compared to SGD alone (2nM A71623 18.44 ± 1.557%, $p<0.05$; 10nM A71623 32.12 ± 6.077%, $p<0.001$; 50nM A71623 21.91 ± 4.782%, $p<0.01$). These results indicate better mitochondrial health (with 2 and 10nM treatment) and less LDH release and therefore membrane rupture (with all concentrations) in cell conditions treated with A71623 compared to heatshock alone. This is confirmed visually by representative photomicrograph of cells in the heatshock alone and with 2nM A71623

treatment conditions as more healthy cells can be seen with A71623. These results show treatment with A71623 can protect undifferentiated cells from induced death by heatshock.

Together with the results from the SGD experiment it can be seen that A71623 can successfully protect these cells from both apoptotic and necrotic cell death and further that 2nM A71623 was the optimum concentration to attain these effects. As such all further work on these cells was carried out at 2nM.

6.4 THE EFFECTS OF A71623 TREATMENT ON NITROSATIVE STRESS

Nitrosative stress (NS) occurs in the body in response to excess RNS, and is linked to pathogenesis in several neurodegenerative diseases (Togo, Katsuse and Iseki, 2004; Colombo *et al.*, 2012; Khandare, Ankulu and Aparna, 2013; Tripathy, Chakraborty and Mohanakumar, 2015). Overproduction of NO in particular can be neurotoxic (Calabrese *et al.*, 2007). As such I aimed to explore if the protective action of CCK1R agonism was caused by modulation of NS.

6.4.1 A71623 and nNOS inhibition may modulate NO under SGD conditions

In order to measure the ability of CCK1R agonism by A71623 to modulate NS undifferentiated SHSY-5Y cells underwent SGD for 24h and were treated with 2nM A71623 and three inhibitors of nitric oxide synthase (NOS). These inhibitors were 100µM N-(1-lminoethyl)-L-ornithnie dihydrochloride (L-NIO, eNOS inhibitor), 10µM S-methyl-L-thiocitrulline (SMTC, nNOS inhibitor) and 10µM S-methylisothiourea (SMUT, iNOS inhibitor). These concentrations were chosen based on previous research (Wang, 1999; Law, Gauthier and Quirion, 2001; Brzozowski *et al.*, 2011). To detect changes in NS cells were stained with the NO detecting fluroescent dye DAF-2DA prior to imaging. At least two coverslips were made for each treatment on each plate down and at least 7 images taken from random fields of view for each coverslip. Cells were used from three separate plate downs. Images were analysed for corrected total cell fluorescence and viability was determined using LDH assay (**Figure 30**).

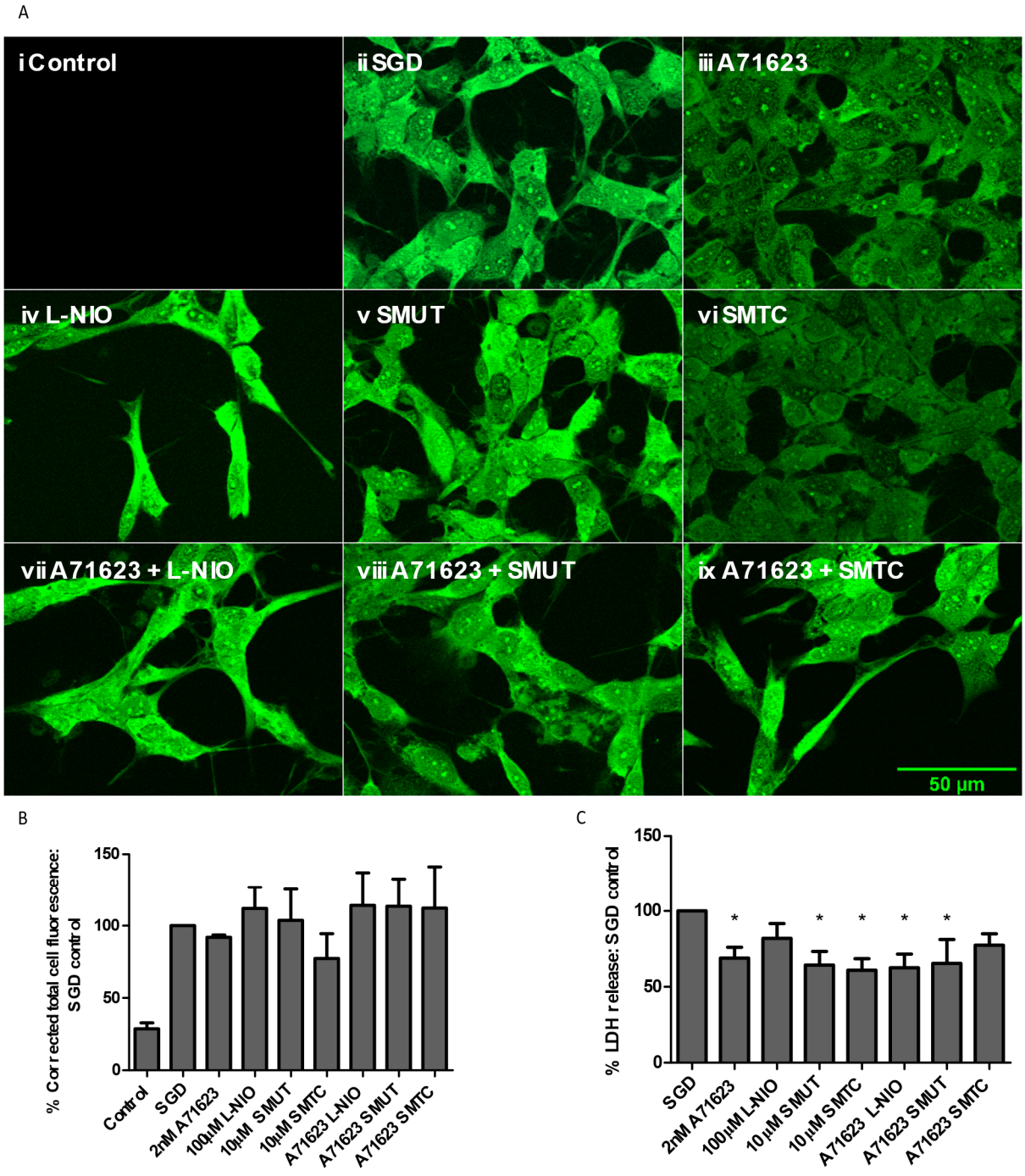

Figure 30. Modulation of NO in SGD treated cells. A – Fluorescent images taken at x63 magnification, show undifferentiated SHSY-5Y cells stained with DAF2-DA and treated for 24h with 10% serum (i), SGD (ii), SGD + 2nM A71623 (iii) SGD + 100µM L-NIO (iv), SGD + 10µM SMUT (v), SGD + 10µM SMTC (vi), SGD + 2nM A71623 + 100µM L-NIO (vii), SGD + 2nM A71623 + 10µM SMUT (viii), SGD + 2nM A71623 + 10µM SMTC (ix). B – The graph shows mean +SEM % corrected total cell fluorescence relative to the SGD condition as measured from the DAF2 treated cells ($F_{(8,18)}$=2.51, p=0.050, n=3). C – Graph shows mean + SEM % LDH release relative to SGD control for cells treated with SGD, A71623 and NOS inhibitors ($H_{(7)}$=16.42, p=0.0216, n=8). Significant relative to SGD control is denoted by asterisks.

The DAF-2DA stained fluorescent images indicate that control, 2nM A71623 and 10µM SMTC treated cells have lower NO present in comparison to the SGD treated cells alone. This trend is

supported by the trend in the corrected total cell fluorescent measures and an overall significant effect is found ($p=0.0500$; $n=3$) but Tukey's multiple comparison test could not identify where this difference lay. From the viability data it can be seen that LDH release was decreased in the 2nM A71623 alone (26.63 ± 7.088%), 10µM SMUT alone (27.25 ± 8.985%), 10µM SMTC alone (29.79 ± 7.618%), A71623 with L-NIO (28.31 ± 8.973%) and A71623 with SMUT (26.44 ± 15.820%) conditions compared to the SGD condition alone ($p<0.05$ for all; $n=8$).

6.4.2 Heatshock does not increase NS in undifferentiated SHSY-5Y cells

Modulation of NS was also examined under heatshock conditions. Undifferentiated SHSY-5Y cells plated on coverslips were heatshocked for 2 minutes then treated with 2nM A71623 and the NOS inhibitors (see 6.4.1) for 24hrs prior to staining with DAF-2DA for NO detection. The control condition was plated in a separate dish but was treated in the same manner to the heatshocked cells other than being exposed to the 60°C water bath. Cells were then imaged, and intensity of green fluorescence measured (Figure 31).

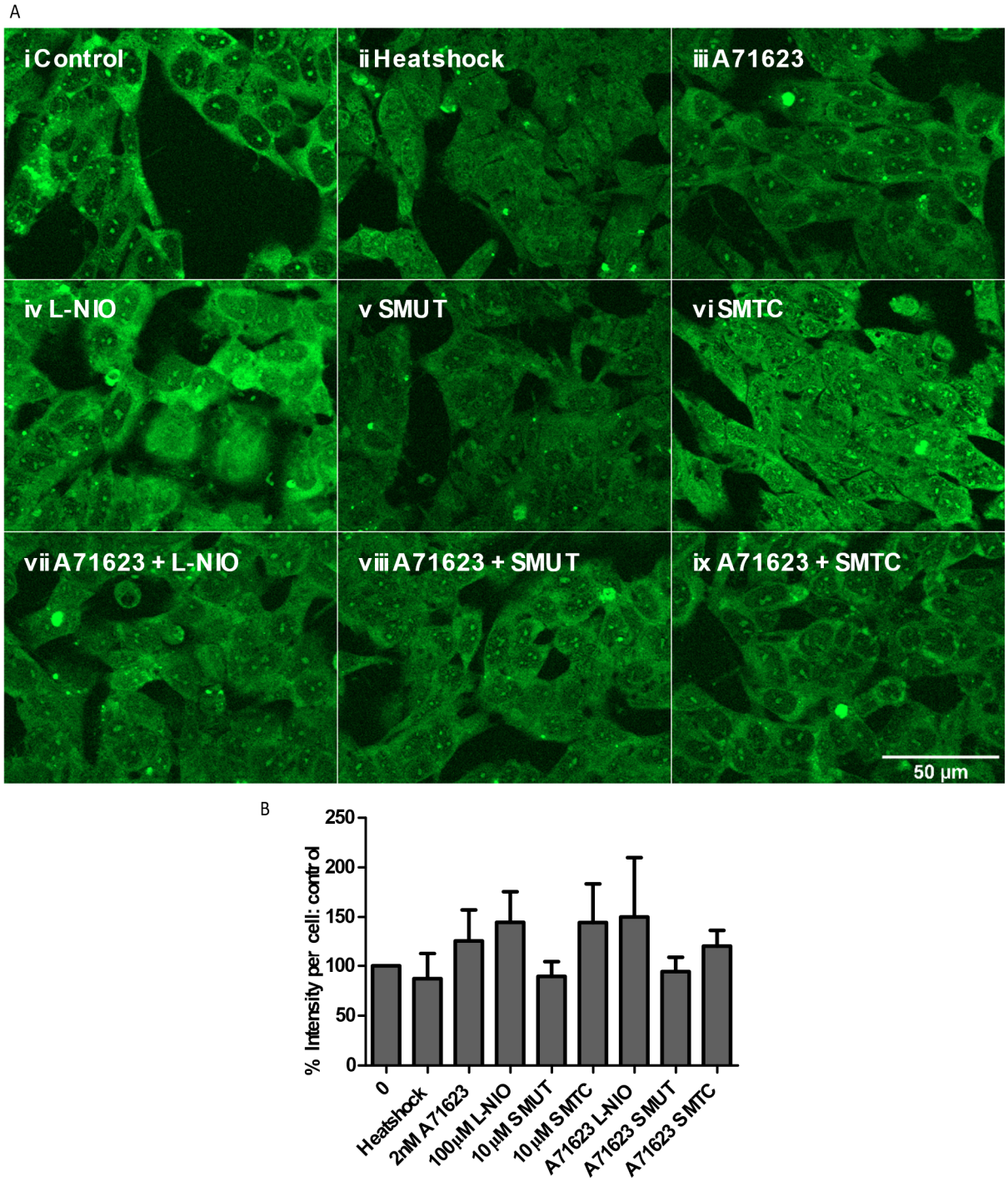

Figure 31. Heatshock did not increase NO production in SHSY-5Y cells. A – Fluorescent images show undifferentiated SHSY-5Y cells stained with DAF2-DA and treated for 24h with 10% serum (i), heatshock (ii), heatshock + 2nM A71623 (iii) heatshock + 100µM L-NIO (iv), heatshock + 10µM SMUT (v), heatshock + 10µM SMTC (vi), heatshock + 2nM A71623 + 100µM L-NIO (vii), heatshock + 2nM A71623 + 10µM SMUT (viii), heatshock + 2nM A71623 + 10µM SMTC (ix). B – The graph shows mean +SEM % intensity per cell relative to the control condition as measured from the DAF2 treated cells ($H_{(8)}$=6.157, p=0.6297, n=8).

The DAF-2DA stained fluorescent images indicate that heatshock treatment alone did not increase NO production in the undifferentiated SHSY-5Y cells. This is supported by the intensity

measures which showed no significant differences between any of the treatment groups (p=0.6297; n=8).

6.4.3 A71623 can downregulate iNOS

In order to assess if CCK1R agonism modulated the expression of NOS isoforms, undifferentiated SHSY-5Y cells were grown and treated for 24h with 2nM A71623 or maintained under control conditions. Protein was extracted from these cultures and after Bradford assay to determine protein concentration, ELISA assays were performed using NOS1 (nNOS), NOS2 (iNOS) and NOS3 antibodies (eNOS) with anti-rabbit secondaries (see **Table 3** for antibody concentrations; **Figure 32**).

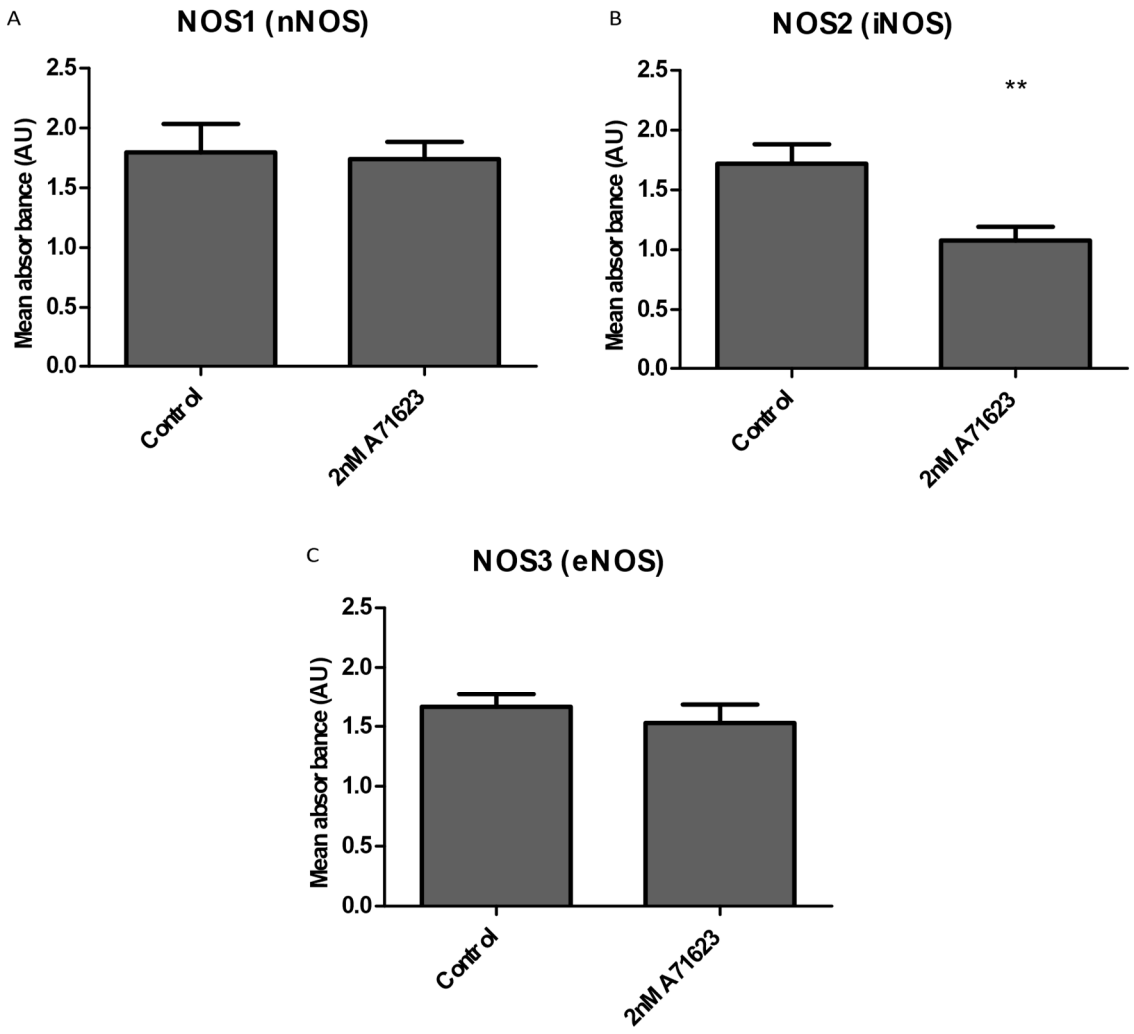

Figure 32. Expression of NOS in A71623 treated cells. Graphs show Mean + SEM absorbance. Treatment of cells with 2nM A71623 for 24h did not affect NOS1 (A: $t_{(4)}$=0.1609, p=0.880, n=5) or NOS3

(C; $t_{(4)}$=0.5731, p=0.5972, n=5) levels but significantly reduced NOS2 levels (B: $t_{(4)}$=4.745, p=0.0090, n=5).

The NOS 1 (p=0.8800) and NOS 3 (p=0.5972) data indicated no significant difference in NOS levels between control and A71623 treated cells. However, for NOS 2, cells treated with A71623 were found to have significantly decreased NOS 2 levels than untreated controls (0.642 ± 0.1142, p=0.0090), indicating that A71623 treatment decreases iNOS expression (n=5 for all).

6.5 A71623 HAS NO IMPACT ON OXIDATIVE STRESS

Another method through which A71623 could be counteracting the effects of SGD and heatshock is through modulation of OS. An overproduction of ROS leads to OS in a biological system which leads to damage of proteins, lipids and DNA (Feeney and Schöneich, 2012; Ivanova and Yankova, 2013) and is linked with ageing and neurodegeneration (Grimm and Eckert, 2017; Nalivaeva and Turner, 2017). As such OS in our cultures was explored by detection of ROS.

6.5.1 SGD but not heatshock increases superoxide and A71623 does not modulate this.

An important ROS is superoxide ($O_2^{\cdot-}$) which is produced in the ETC of the mitochondria and during the immune response to invading pathogens (Guzik, Korbut and Adamek-Guzik, 2003). It is usually converted to water and oxygen by SOD, but the system is imperfect as such an overproduction of superoxide can lead to OS, cell damage and death (**Figure 1**). Therefore, the NBT assay was used to detect the presence of superoxide in cultures treated with either SGD or heatshock and 2nM A71623 for 24h (Figure 33).

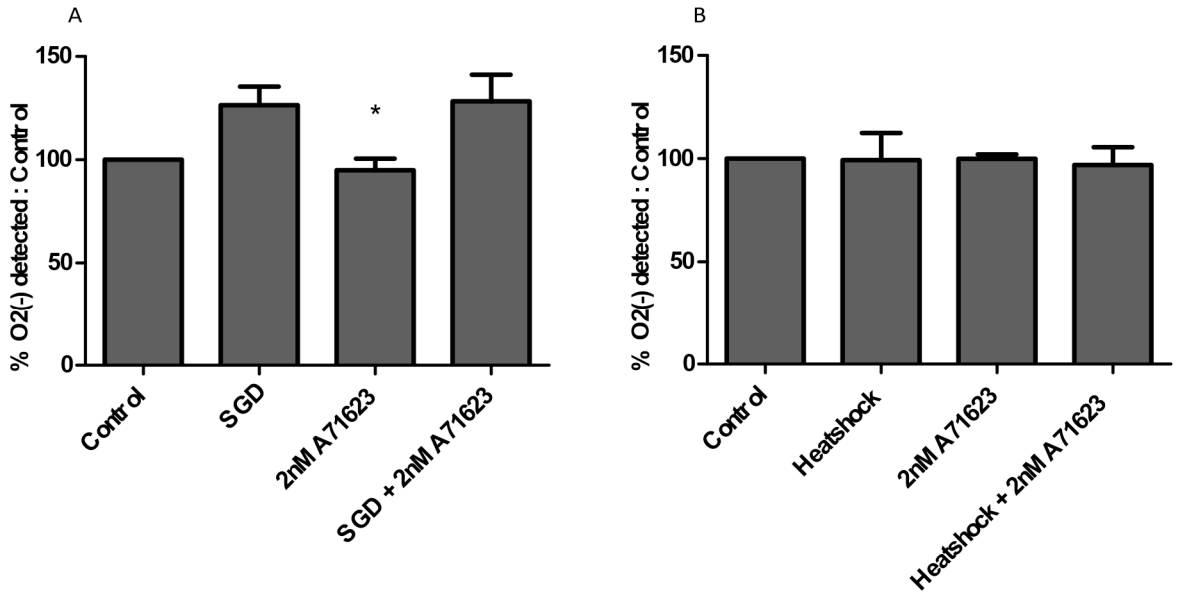

Figure 33. A71623 does not modulate superoxide production. Graphs show mean + SEM percent superoxide detection relative to untreated control condition. A – SGD treatment alone caused greater superoxide production in cultures compared to 2nM A71623 treatment alone, but A71623 could not prevent increased superoxide production under SGD conditions ($H_{(3)}$=10.08, p=0.0179, n=6). Significance relative to the SGD condition is denoted by an asterisk. B – Heatshock treatment for 24h did not increase superoxide production and 2nM A71623 had no other effects ($H_{(3)}$=3.432, p=0.3297, n=6).

In the SGD experiment (n=6) 2nM A71623 alone significantly decreased superoxide compared to SGD treatment alone (22.61 ± 5.626%, $p<0.05$). This shows that the SGD condition caused an increase in superoxide and 2nM A71623 treatment alone did not. However, the 2nM A71623 and SGD combined treatment produced similar levels of superoxide to SGD alone showing that A71623 could not prevent this increase. For the heatshock data no significant differences were found (p=0.3297; n=6). This shows that heatshock alone does not increase superoxide in these cells and A71623 had no further modulatory effects.

6.5.2 SGD increases oxidative stress and A71623 does not ameliorate this effect

The H_2DCFDA dye is a more general measure of ROS as it has less specificity in its reaction than the NBT assay. As the SGD was able to increase the ROS superoxide in our cultures it was explored whether A71623 could modulate a more generalised OS effect under these conditions. As such cells underwent SGD for 24h with or without 2nM A71623 and were stained with the H_2DCFDA dye and corrected total cell fluorescence measured (n=2, **Figure 34**).

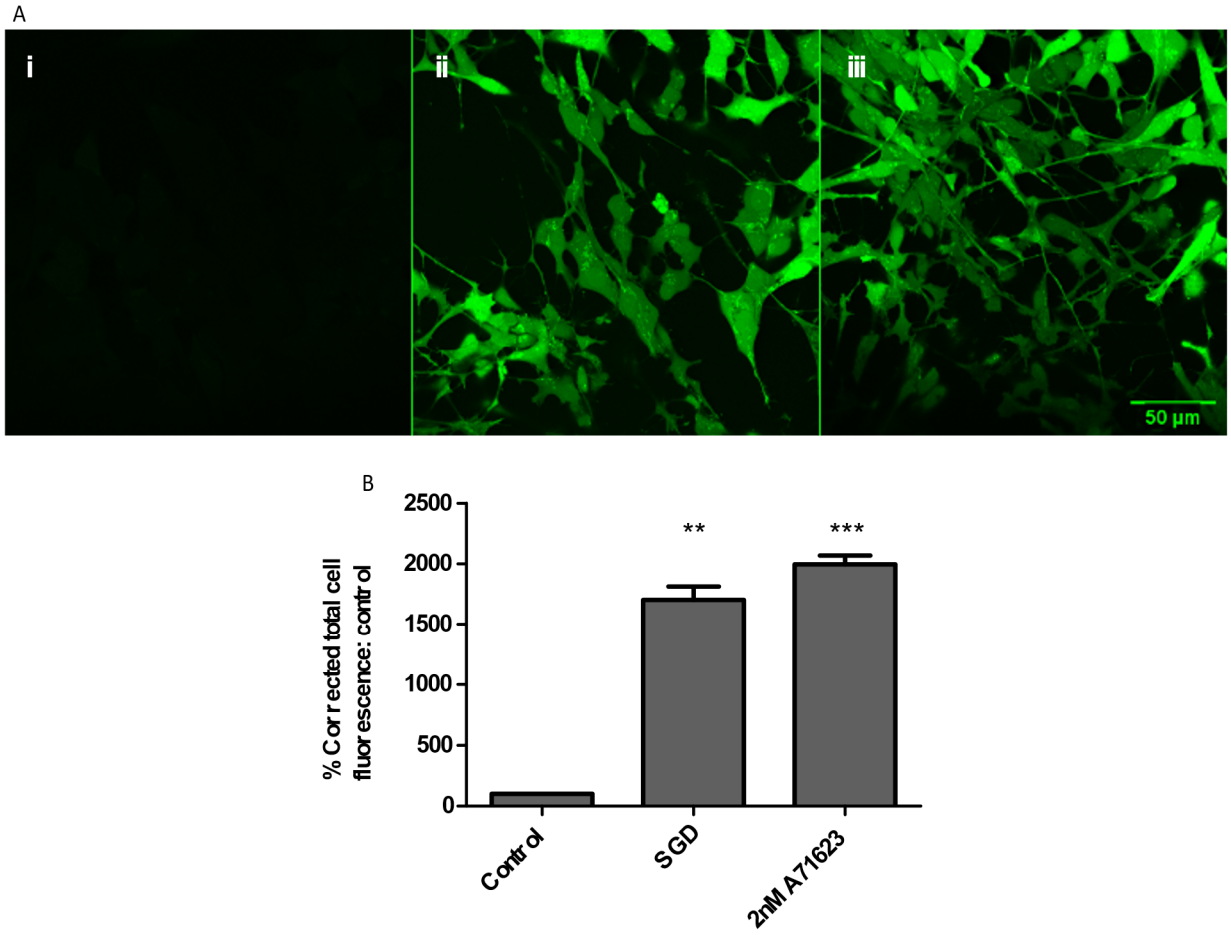

Figure 34. SGD increased oxidative stress in undifferentiated SHSY-5Y cells and A71623 does not prevent this. A – Representative images of cells stained with H_2DCFDA under control (i), SGD (ii) and SGD + 2nM A71623 (iii). Green staining is visibly greater in SGD and SGD + 2nM A71623 conditions compared to control. B – Graph showing mean + SEM percent corrected total cell fluorescence relative to control condition as measured from cells stained with H_2DCFDA ($F_{(2,3)}$=182.6, p=0.0007, n=2). Significance relative to control is denoted with asterisks.

The images taken from the H_2DCFDA stained cells show more green staining in SGD and SGD with 2nM A71623 conditions compared to control. Similarly, CTCF shows SGD (1602 ± 109.5%, p<0.01) and SGD + 2nM A71623 (1896 ± 71.50%, p<0.001) conditions had significantly greater fluorescence than control condition. This demonstrates SGD was able to increase oxidative stress in these cultures and this could not be prevented by A71623 treatment.

6.6 CCK1R AGONISM DOES NOT PROTECT HT22 CELLS FROM TOTAL SERUM DEPRIVATION

As CCK1R agonism shows protective abilities in undifferentiated SHSY-5Y cell line it was investigated whether it has potential protective abilities in the mouse hippocampal cell line, HT22.

Following the establishment of complete serum removal as an effective method of inducing cell death in these cultures (see 5.7), HT22 cells were treated with a range of A71623 concentrations from 0.4-50nM for 24hr and viability measured by CV and LDH assays (Figure 35).

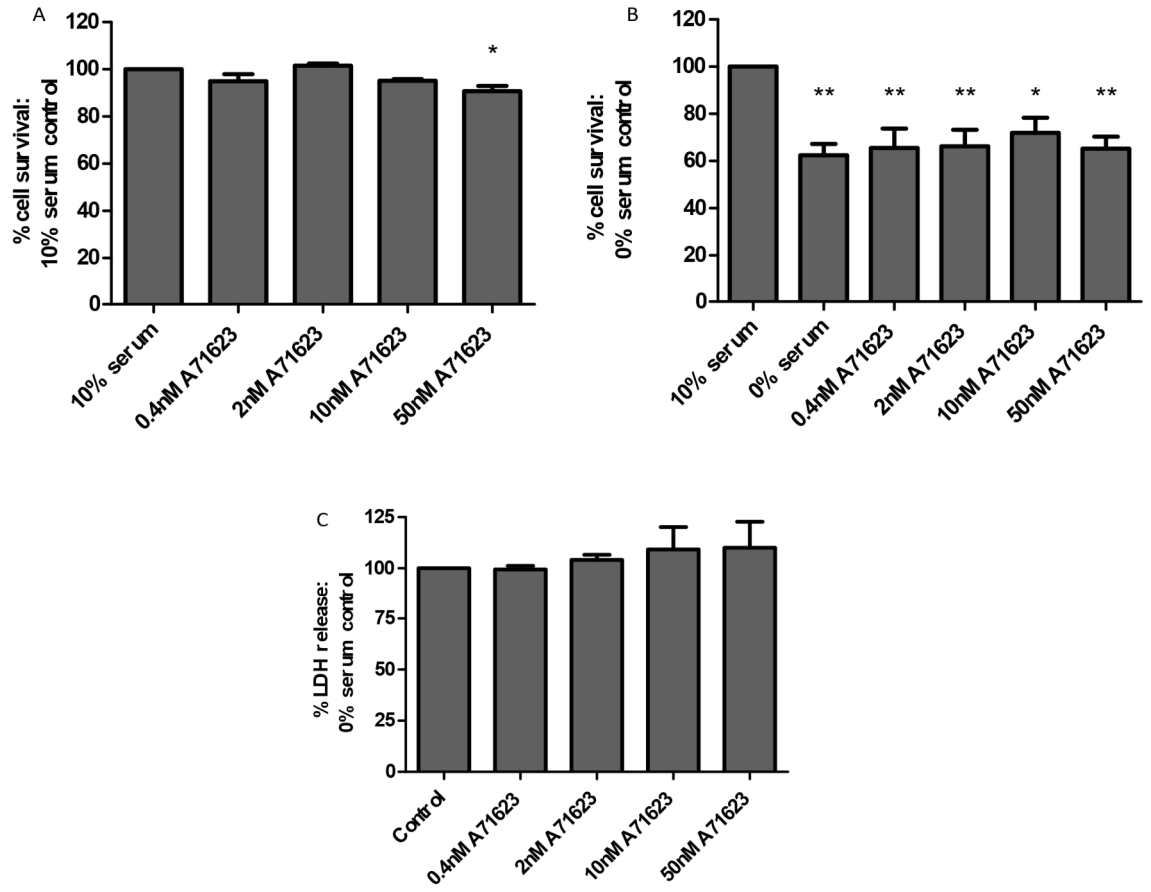

Figure 35. Agonism of the CCK1 receptor via A71623 does not protect HT22 cells from full serum deprivation. A - The graph shows mean + SEM % cell survival relative to the 10% serum control cells as determined by crystal violet assay. Significant decrease in cell number was found with the highest concentration of A71623 ($F_{(4,15)}$=6.041, p=0.0042, n=4). B – Graph shows mean + SEM % cell survival of 0% serum containing conditions relative to the 10% serum control cells as determined by crystal violet assay ($F_{(5,18)}$=5.785, p=0.0024, n=4). C - The graph shows mean + SEM % LDH release relative to 0% serum control. No significant difference was found between conditions ($H_{(4)}$=2.346, p=0.6724, n=4). Significance is denoted by asterisks.

The CV data (n=4) for the 10% serum containing conditions showed that the 50nM A71623 condition had significantly decreased cell number compared to the 10% control (9.251 ± 2.161%, p<0.05). This demonstrates that under healthy conditions the highest concentration of A71623 used on these cells was toxic. There was also a significant difference between 2nM and 50nM A71623

treatments (10.76 ± 2.161%, p<0.01) demonstrating that this lower concentration was not toxic as it had significantly higher cell numbers.

The CV data for the 0% serum containing conditions had significantly reduced cell survival than the 10% condition (0% serum 37.5 ± 4.718%, p<0.01; 0.4nM A71623 34.43 ± 8.166%, p<0.01; 2nM A71623 33.77 ± 7.045%, p<0.01; 10nM A71623 28.09 ± 6.447%, p<0.05; 50nM A71623 34.72 ± 5.110%, p<0.01). This shows that no concentration of A71623 used could prevent cell number decrease caused by total serum withdrawal.

The LDH data (n=4) showed no significant difference between conditions (p=0.6724). Further showing no protective effect for CCK1R agonism by A71623 in these conditions.

It was considered if CCK1R expression might change with differentiation and hence if the neuroprotective abilities of A71623 should be explored further post differentiation. In order to determine if further exploration was warranted protein was extracted from undifferentiated and differentiated HT22 cells to run an ELISA for CCK1R (see **Table 3** and **Figure 36**).

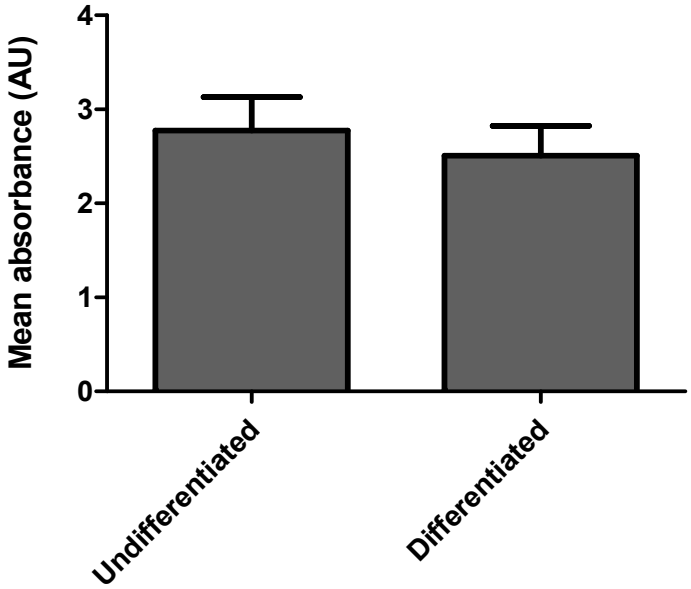

Figure 36. CCK1R expression in undifferentiated and differentiated HT22 cells. Graph shows mean +SEM absorbance in the ELISA. No differences in CCK1R expression levels in undifferentiated compared with differentiated HT22 cells were found ($t_{(4)}$=0.8325, p=0.4519, n=5).

The ELISA assay shows no significant difference in CCKAR expression between undifferentiated and differentiated HT22 cells (p=0.4519; n=5). CCK1R expression is similar in the undifferentiated and differentiated cell line indicating no further exploration of the differentiated state is warranted.

6.7 CCK1R IS EXPRESSED IN DIFFERENTIATED SHSY-5Y CELLS

Having found that CCK1R agonism in undifferentiated SHSY-5Y cells is protective against cell death this warranted further exploration as to A71623's abilities in fully differentiated SHSY-5Y. First the presence of the CCK1R in the differentiated cell line was confirmed and compared to the undifferentiated cells. This was done to ensure the receptor was still present and hence A71623 would have a site of action in these cells and to identify changes in receptor expression. Therefore untreated cultures were used at 48h post plate down (undifferentiated), after 5 days in retinoic acid (differentiated) and at 1 week post-differentiated (7 days in mitotic inhibitor medium) and were used for ELISA assay (**Figure 27Figure 37**A) and ICC (**Figure 37**B). For the ELISA assay protein was extracted from an n of 8 experimental repeats and Bradford assays were run to determine equal loading. CCK1R and α-tubulin primary antibodies with anti-rabbit and anti-mouse secondaries were used for the ELISA and CCK1R with anti-rabbit fluorescein secondary were used for the ICC as stated (See **Table 3**).

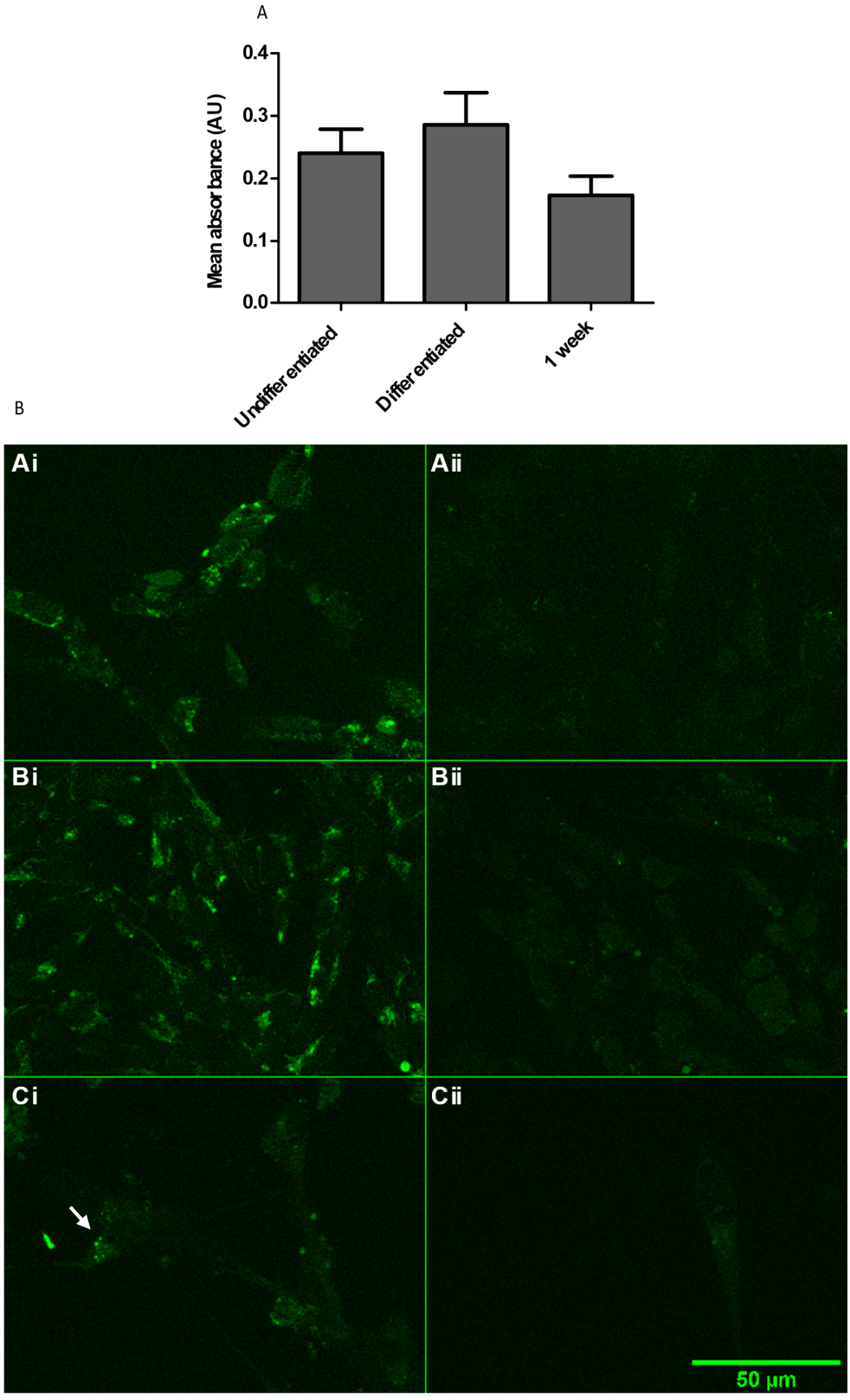

Figure 37. CCK1R is expressed in differentiated SHSY-5Y cells and expression levels do not significantly change with differentiation. A- graph shows mean + SEM absorbance, as detected by ELISA, of CCK1R expression in undifferentiated and differentiated SHSY-5Y cells. No significant changes

in receptor expression were found ($F_{(2,7)}$=3.034, p=0.0804, n=8). B – Fluorescent images of undifferentiated (A), differentiated (B) and 1-week post differentiation (C) SHSY-5Y cell line are shown labelled with a CCK1R primary antibody and Fluorescein secondary (i) and labelled with Fluorescein secondary in the absence of the primary antibody (ii). Greater staining in the primary containing conditions indicates the presence of the receptor in this cell line. White arrows indicate instances of CCK1R staining in the 1-week post-differentiation image.

The ELISA assay (n=8) demonstrated the CCK1R was expressed in this cell line at all stages of differentiation used in this chapter. There was no significant difference in CCK1R expression between the differentiation states (p=0.0804). Levels of the CCK1R expression did not change significantly in the SHSY-5Y cells during differentiation. However, results from the immunocytochemistry suggest a slightly different pattern. Staining was observed for all differentiation states but staining at 1-week post differentiation (n=2) visually appears more sparse in comparison to undifferentiated (n=6) and differentiated (n=4) cultures.

6.8 CCK1R AGONISM BY A71623 PROTECTS AGAINST NEURONAL DEATH IN VITRO

Given that CCK1R agonism by A71623 could protect against cell death in undifferentiated cells I wanted to explore if these protective abilities continued post-differentiation in a neuronal phenotype. As the overall aim is to establish if CCK1R agonism has potential in AD treatment, and this is a disease that effects mature neurons, investigating our compounds effects on this cell type was the next logical step. Differentiated SHSY-5Y cells were therefore treated with 0.4, 2 and 10nM A71623 and either copper chloride (Cu) or Aβ in the following experiments. These concentrations were chosen based on 2nM being optimal in the undifferentiated cultures, therefore I chose one concentration five-fold higher and one five-fold lower than this for a dose response.

6.8.1 A71623 prevents decreased mitochondrial activity and membrane rupture caused by copper chloride

As mentioned previously heavy metals have been implicated in neurodegenerative diseases and as such divalent metal ions are used to model this *in vitro* (see 5.3.1). Cytotoxicity in this study was induced by 10μM copper chloride, MTT, LDH and CV assays were used to determine cell viability after 96 hours and representative photomicrographs taken to demonstrate changes visually (**Figure 38**).

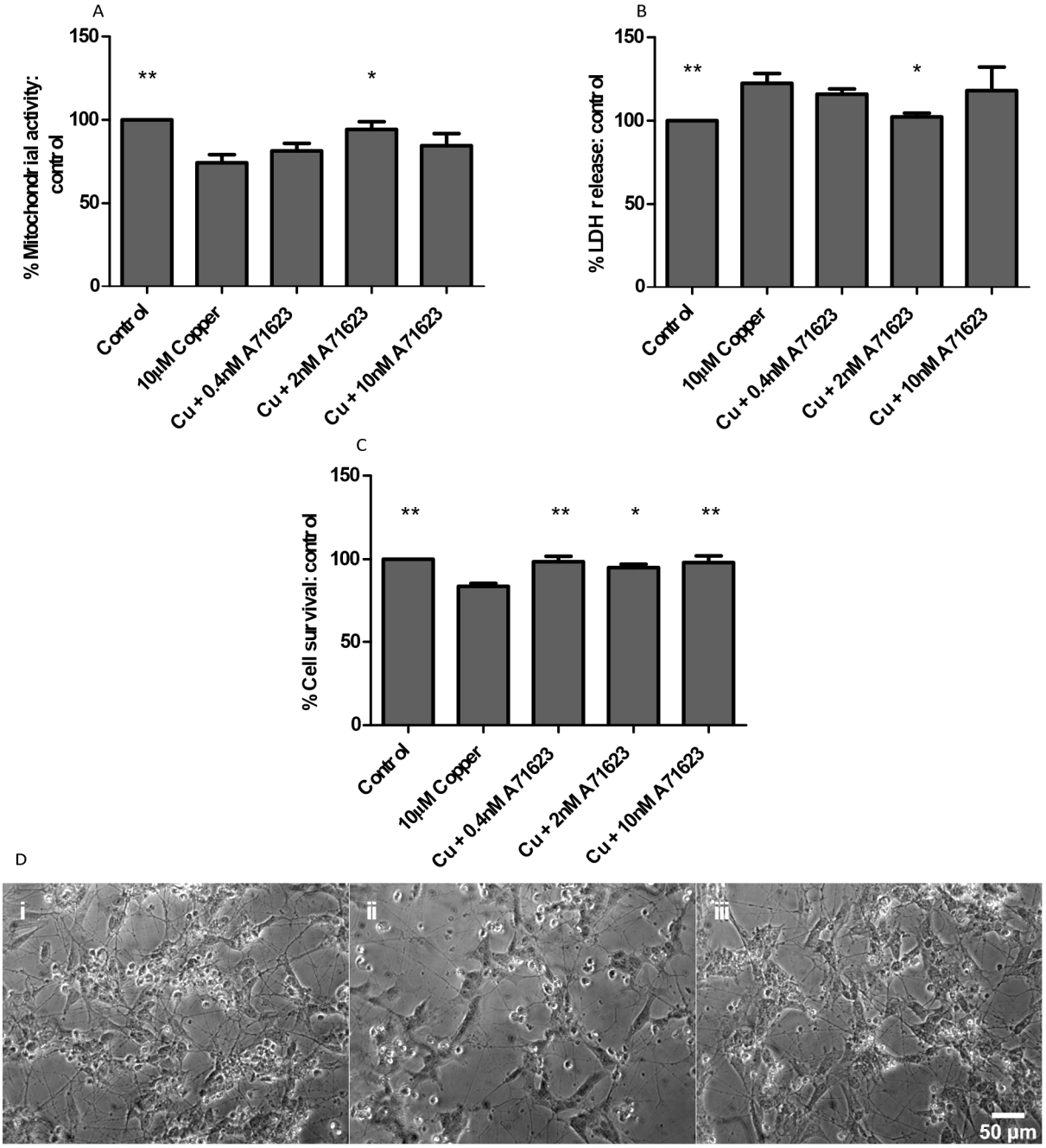

Figure 38. A71623 was able to protect against copper chloride induced cell death. Graphs show mean + SEM. Significance relative to Cu condition indicated by asterisks. A – Results of MTT assays show that 2nM A71623 had significantly higher mitochondrial activity compared to Cu treated cultures alone ($F_{(4,30)}$=4.397, p=0.0065, n=7). B – The LDH assay demonstrated significantly lower LDH release in the 2nM A71623 and control conditions relative to copper treatment ($H_{(4)}$=16.08, p=0.0029, n=6). C – Cell survival was better in all A71623 treated conditions as measured by CV assay compared to copper treatment alone ($F_{(4,15)}$=6.948, p=0.0023, n=4). D – Shows representative photomicrographs of control (i), 10µM copper (ii) and copper with 2nM A71623 (iii) conditions.

The results of the MTT assay (n=7) demonstrated that control (25.73 ± 0%, p<0.01) and 2nM A71623 (20.02 ± 4.696%, p<0.05) treated cells had significantly increased mitochondrial activity than cultures treated with Cu alone. The LDH assay results (n=5) showed control (22.5 ± 0%, p<0.01) and

2nM A71623 (20.02 ± 2.287%, p<0.05) treated cells had significantly reduced LDH release compared to Cu treatment alone. The CV assay (n=4) showed all conditions (control: 16.73 ± 0%, p<0.01; 0.4nM A71623: 15.29 ± 3.250%, p<0.01; 2nM A71623: 11.85 ± 2.002%, p<0.05; 10nM A71623: 14.84 ± 3.937%, p<0.01) had significantly higher cell survival compared to Cu treatment alone. Finally, representative photomicrographs demonstrate greater cell number and more processes in control and 2nM A71623 treated conditions relative to copper alone. Together these results demonstrate that 2nM A71623 was consistently able to produce a protective effect against copper induced toxicity.

6.8.2 A71623 prevents decreased mitochondrial activity and membrane rupture caused by $A\beta_{1-42}$

$A\beta_{1-42}$ is integral to plaque formation in AD and is toxic to neurons, as such it can be used as and *in vitro* model of AD (see 3.3 and 5.3.2). To test the abilities of CCK1R agonism by A71623 to be protective against $A\beta_{1-42}$, differentiated SHSY-5Y cells were treated with 0.4, 2 and 10nM A71623 for 96

hours, MTT, LDH and CV assays were conducted to measure cell viability and representative photomicropgraphs taken to demonstrate changes visually (**Figure 39**).

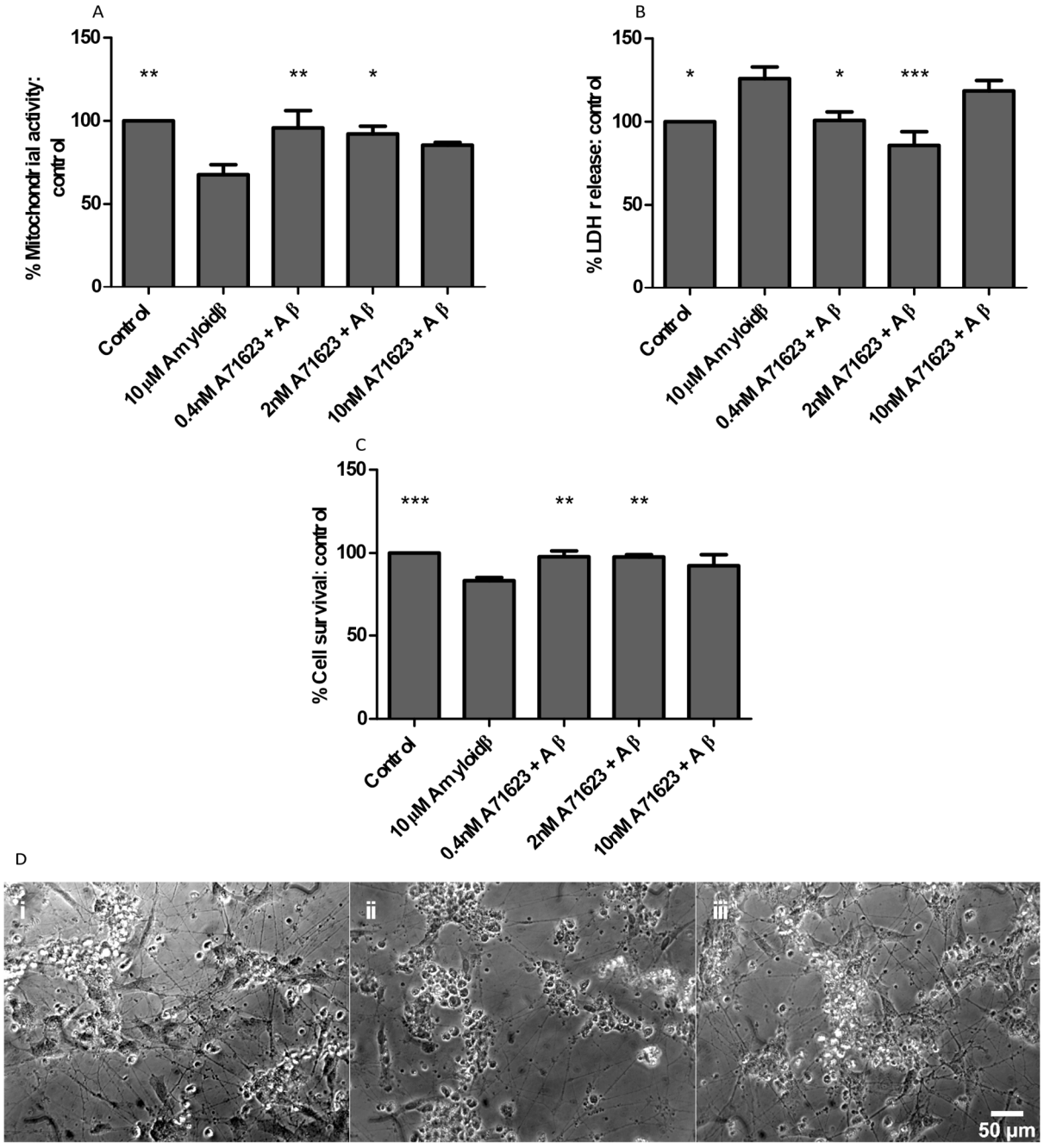

Figure 39. A71623 can protect cells from death induced by Aβ. Graphs show mean + SEM. Significance relative to Aβ condition indicated by asterisks. A- Results of MTT assays show that 0.4 and 2nM A71623 had significantly higher mitochondrial activity compared to Aβ treated cultures alone ($F_{(4,20)}$=4.869, p=0.0066, n=5). B – LDH assay results show 2 and 0.4nM A71623 had significantly lower LDH release compared to Aβ treatment alone ($F_{(4,19)}$=6.985, p=0.0012, n=5). C- Higher cell survival was seen in 0.4 and 2nM A71623 treated conditions compared to Aβ alone as deduced by CV assay ($F_{(4,23)}$=6.233, p=0.0015, n=6). D – Representative photomicrographs of control (i), 10µM Aβ (ii) and 2nM A71623 with Aβ (iii) are shown.

The results from the MTT assay (n=5) showed that control (32.31 ± 0%, $p<0.01$), 0.4nM A71623 (28.18 ± 10.31%, $p<0.01$) and 2nM A71623 (24.54 ± 4.527%, $p<0.05$) had significantly increased mitochondrial activity relative to cultures treated with Aβ alone. The LDH data found control (25.91 ± 0%, $p<0.05$), 0.4nM A71623 (25.12 ± 5.173%, $p<0.05$) and 2nM A71623 (40.14 ± 8.289%, $p<0.001$) had significantly reduced LDH release compared to Aβ treatment alone. Further the CV assay results (n=5) shows control (17.03 ± 0%, $p<0.001$), 0.4nM A71623 (14.91 ± 3.424%, $p<0.01$) and 2nM A71623 (14.73 ± 1.399%, $p<0.01$) conditions all had increased cell survival compared to Aβ treatment alone. Finally, representative photomicrographs visually demonstrate greater cell number in control and 2nM A71623 treated cells compared to Aβ treated cultures. This data demonstrates both 0.4nM and 2nM A71623 can produced protective effects against Aβ in culture.

6.9 CCK1R AGONISM BY A71623 ACTIVATES STAT3 BUT NOT AKT PRO-SURVIVAL PATHWAYS

CCK1R is known to signal by the PI3K/Akt pathway (Zhou *et al.*, 2014) and it has been suggested that CCK's effects on cell proliferation are mediated via this same pathway (Buscail *et al.*, 1995). As such it was explored whether the neuroprotective effects seen with A71623 were mediated via this pathway. Additionally, previous work has shown CCK-8 can activate the STAT3 pathway at concentrations ranging from 0.1-100nM (Heldsinger *et al.*, 2011) this is another pro-survival pathway which may provide an alternative route of action. To investigate these pathways protein was extracted from differentiated SHSY-5Y cells which were either untreated or treated with 2nM A71623 for 96 hours. ELISA assays were then run for pSTAT3 and pAkt (see **Table 3** for antibody concentrations; **Figure 40**).

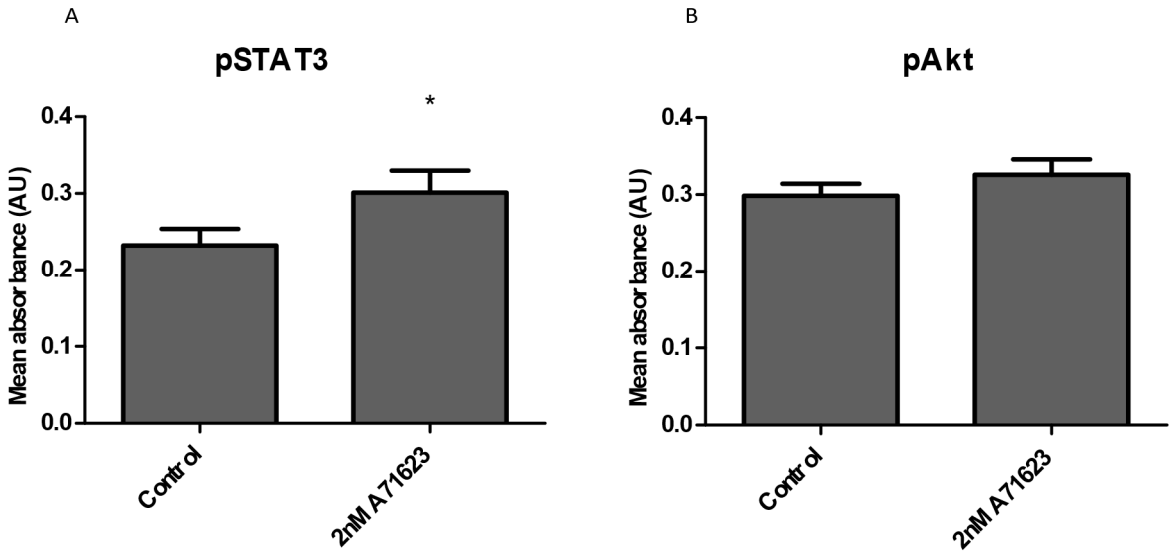

Figure 40. A71623's neuroprotective effects are mediated via the STAT3 but not the Akt signalling pathway in differentiated SHSY-5Y cells. The graphs show mean + SEM absorbance from ELISA assays. A – Show pSTAT3 levels in protein extracts from differentiated SHSY-5Y cells untreated or treated with 2nM A71623 ($t_{(6)}$=3.684, p=0.0103, n=7). B – Shows pAkt levels in protein extracts from differentiated SHSY-5Y cells which were untreated or treated with 2nM A71623 ($t_{(7)}$=1.232, p=0.2579, n=8). Significance relative to the untreated control is denoted by asterisks.

The ELISA shows A71623 activates the pSTAT3 pathway in differentiated SHSY-5Y cells, as there was a significant increase in pSTAT3 levels from untreated to A71623 treated cells (0.0692 ± 0.02845, p=0.0103, n=7). The pAkt data showed no significant difference (p=0.2579, n=8), therefore A71623 does not activate this pathway in differentiated SHSY-5Y cells after 96h treatment.

6.10 A71623 MODULATES P-TAU BUT NOT OTHER KNOWN BIOMARKERS OF ALZHEIMER'S DISEASE, SIGNIFICANTLY, IN CULTURES TREATED WITH LOW-DOSE AB_{1-42}

In order to test whether A71623 could modulate biomarkers of AD, differentiated SHSY-5Y cultures were treated with 1µM $A\beta_{1-42}$ for five days as well as 2nM A71623 with or without $A\beta_{1-42}$. Protein was extracted from these cultures and ELISAs were run for AD relevant biomarkers: p-tau, ABAD, APP, endophilin 1 and presenilin 1 as previously described (see 5.5). For this experiment a total of 11 experimental repeats were made however only a maximum n of 6 were used, following the previously described inclusion criteria of increased p-tau in the Aβ alone treated condition (see **Table 3** for antibody concentrations; **Figure 41**).

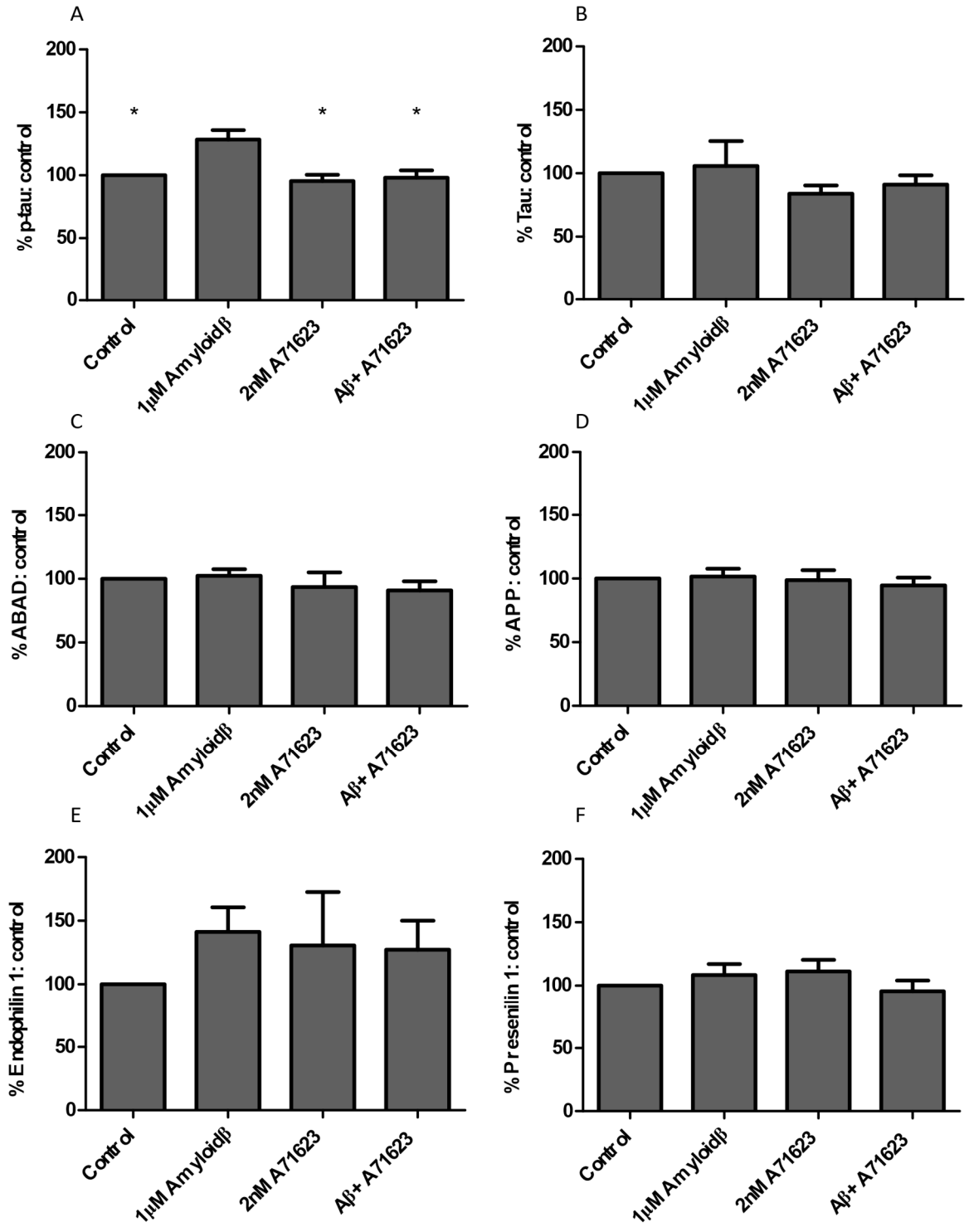

Figure 41. ELISAs for AD biomarkers in cultures treated with Aβ and A71623. The graphs show mean percentage antibody relative to control + SEM. A – Shows that Aβ significantly increased p-tau and A71623 was able to ameliorate this effect ($H_{(3)}$=12.23, p=0.0066, n=6). Significance relative to the Aβ treated condition is denoted with an asterisk. B – A71623 may have slightly decreased tau levels in these cultures but little change can be seen in tau levels with other treatments ($H_{(3)}$=5.815, p=0.121, n=6). C - Whilst Aβ treatment had little effect on ABAD levels, there is a suggested decrease in ABAD with A71623 treatment both with and without Aβ ($H_{(3)}$=1.500, p=0.6823, n=4). D – Shows no variation in APP levels ($H_{(3)}$=0.7484, p=0.8618, n=5). E – All treatments show a suggested increase in endophilin 1 relative to control ($H_{(3)}$=3.313, p=0.3458, n=4). F – Shows there may be a trend towards decreased

presenilin 1 level in the cultures treated with Aβ and A71623 together, but all other conditions appear similar ($H_{(3)}$=1.862, p=0.6015, n=5).

Decreases in p-tau between control (11.67 ± 0%, p<0.05), 2nM A71623 (11.83 ± 4.882%, p<0.05) and Ab + 2nM A71623 (11.17 ± 5.863%, p<0.05) relative to 1μM Aβ were found. No other significant differences occurred (tau p=0.1210; ABAD p=0.6823; APP p=0.8618; Endophilin 1 p=0.3458; Presenilin 1 p=0.6015). These data indicate that 1μM Aβ significantly upregulated p-tau and 2nM A71623 could modulate this effect. However, this concentration of Aβ could not significantly upregulate any of the other biomarkers in these cultures.

6.11 CCK1R AGONISM BY A71623 DOES NOT SIGNIFICANTLY IMPROVE PERFORMANCE IN AN EPISODIC-LIKE MEMORY TASK RELATIVE TO CONTROLS.

Given that increasing CCK has previously been implicated in improving memory (Voits *et al.*, 2001; Sadeghi, Reisi and Radahmadi, 2017) and a lack of the CCK1R has specifically been linked to memory deficits (Nomoto *et al.*, 1999; Xue-Liang Li *et al.*, 2002; Matsushita *et al.*, 2003), it may follow that agonism of the CCK1R by A71623 can itself improve memory. As A71623 was able to act as a neuroprotective and has shown ability to modulate p-tau increases caused by Aβ, it is of particular interest if A71623 could have memory enhancing effects on an AD-relevant memory task. As such the potential for A71623 to modulate episodic memory was assessed on the OPC memory task, as this is a task that can assess episodic-like memory and hence is relevant to AD (see 5.6). 25 mice were initially used in this experiment (12 in the saline condition and 13 in A71623 condition), however during OPC testing one mouse was found to be significantly smaller than the rest (weight greater than 2 standard deviations below group mean), have a birth defect of the feet, and was rarely performing the memory task, as such this mouse was removed from analysis. Therefore, final numbers of mice in each group were 11 in the saline condition and 13 in the A71623 condition.

6.11.1 All pre-treatment groups performed similarly on the NOR, OP and OC tasks

Mice in both groups performed NOR, OP and OC recognition tasks, each repeated over four days. This allows for assessment of their abilities pre-treatment and to ensure there aren't any pre-

existing differences in the two groups of mice that will impact performance on the OPC memory task (Figure 42). After completing these tasks but before data analysis mice were assigned to their treatment groups in accordance with counterbalancing.

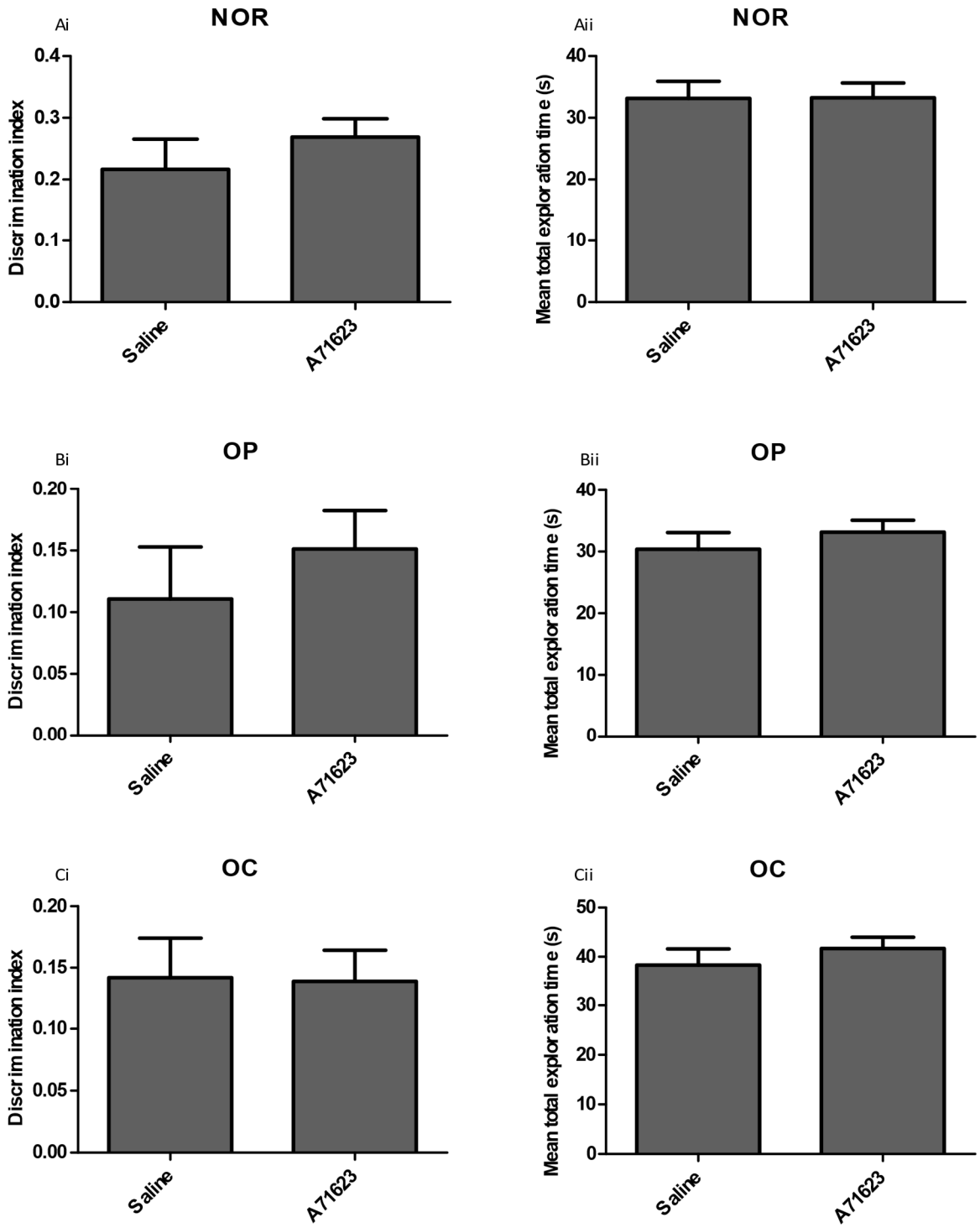

Figure 42. All pre-treatment groups of mice performed similarly on NOR, OP and OC tasks. All graphs show mean + SEM. A – performance on the NOR task measured via discrimination index ($t_{(23)}$=0.9308, p=0.3616, n=13) (i) and total exploration time ($t_{(23)}$=0.0227, p=0.9821, n=13) (ii). B – performance on

the OP task measured via discrimination index ($t_{(23)}$=0.7797, p=0.4435, n=13) (i) and total exploration time ($t_{(23)}$=0.8469, p=0.4058, n=13) (ii). C- performance on the OC task measured via discrimination index ($t_{(23)}$=0.0757, p=0.9403, n=13) (i) and total exploration time ($t_{(23)}$=0.8519, p=0.4030, n=13) (ii).

The d.i. data for the NOR task showed no significant difference in performance between pre-treatment saline and A71623 groups (p=0.3616). One-sample t-tests to a hypothetical mean of 0, showing no preference for either novel or familiar object, showed both pre-treatment saline ($t_{(11)}$=4.430, p=0.0010, n=12) and pre-treatment A71623 ($t_{(12)}$=9.034, p<0.0001, n=13) mice performed above chance. The exploration time data also found no significant difference in exploration times between the two pre-treatment groups (p=0.9821). All animals were able to discriminate between novel and familiar objects with a preference for novel objects and explored similarly.

In the OP task d.i. data showed no difference in performance between the two groups (p=0.4435), both groups performed above chance levels (saline $t_{(11)}$=2.635, p=0.0232, n=12; A71623 $t_{(12)}$=4.845, p=0.0004, n=13) and no difference in total exploration times between pre-treatment groups (unpaired t-test p=0.4058). Animals in both the saline and A71623 pre-treatment groups were able to discriminate novelty of on object in place, explored for similar lengths of time and were of similar ability in this task.

Finally, for the OC task no differences were found between pre-treatment groups performance according to d.i. (p=0.9403) and both groups performed above chance (saline $t_{(11)}$=4.415, p=0.001, n=12; A71623 $t_{(12)}$=5.490, p=0.0001, n=13). Exploration time data showed animals explored similarly (p=0.4030). Both pre-treatment groups were able to discriminate a novel object in context and explored similarly across groups.

6.11.2 Agonism of CCK1R in mice improves performance on an OPC task relative to chance.

After completing NOR, OP and OC testing mice were tested on the OPC task. Mice were injected intraperitoneally with saline or A71623 (10nM/kg) 30 minutes prior to testing to allow time for the drug to circulate through the system and allow for recovery from injection. This concentration was chosen as it has previously been shown to impact food intake in the first 180minutes post i.p. injection, did not

affect locomotion (which is key to this behavioural study) and mice retained their starting weight after 11 days of treatment, though controls gained weight (Asin, Bednarz, Nikkel, Gore and Nadzan, 1992). This shows that this concentration of treatment is high enough to elicit effects within a mouse but not so high as to impact their locomotive behaviour which would impact performance in the memory task. Their performance on the task was assessed post-testing and discrimination index and total exploration times recorded at both 1- and 3-minute time points (Figure 43).

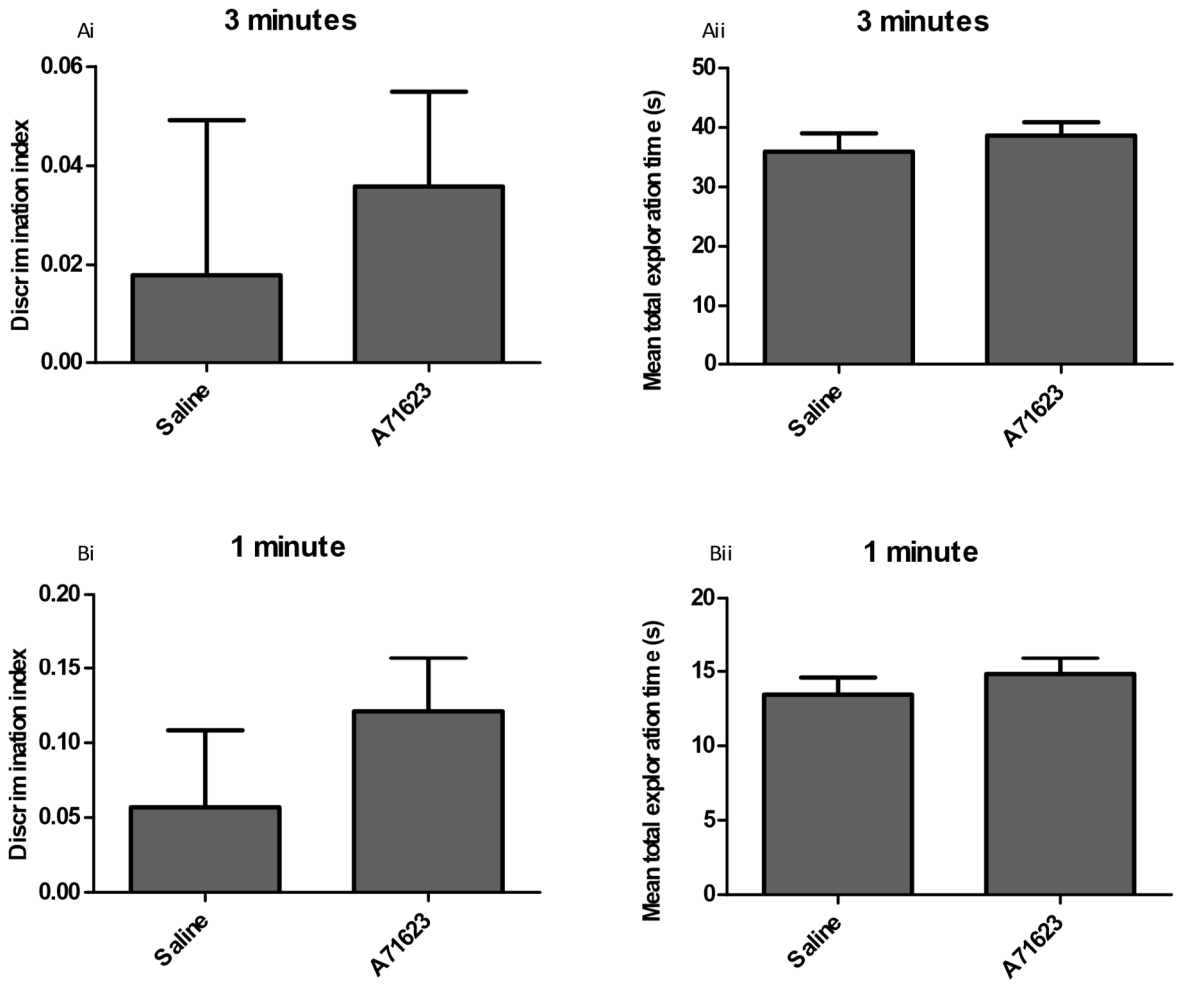

Figure 43 A71623 effects performance on an episodic-like memory task relative to chance. All bar graphs show mean + SEM A – Shows the discrimination index (i) and exploration time (ii) results from the OPC task as measured at the full 3-minute time point, neither group performed above chance and there were no differences in discrimination index ($t_{(23)}$=0.4969, p=0.6240, n=13) or exploration time ($t_{(23)}$=0.7154, p=0.4816, n=13). B – Shows the discrimination index (i) and exploration time (ii) results from the OPC task as measured at the 1-minute time point, only mice treated with A71623 performed above chance in discriminating the novel object but there was no significant difference between discrimination index ($t_{(23)}$=1.046, p=0.3071, n=13) or exploration time ($t_{(23)}$=0.8684, p=0.3945, n=13).

Assessment of the data at the full 3-minute time point (**Figure 43**A) showed no significant difference between saline and A71623 treated mice for d.i. (p=0.6240) and neither group were able to perform above chance on this task (saline $t_{(11)}$=0.5715, p=0.5791, n=12; A71623 $t_{(12)}$=1.871, p=0.086, n=13). The mean exploration time data showed there were no differences between the two groups (t-test p=0.4816). Neither animals treated with saline alone or with A71623 were able to perform the OPC task above chance levels when analysing the 3-minute data. As mentioned earlier (see **5.6.2**) preference for the novel object can significantly decline over the course of testing. As all of these mice could perform each of NOR, OP and OC task above chance it was decided to analyse the 1-minute data as well (**Figure 43**B). The 1-minute d.i. data also showed no significant difference between saline and A71623 treated animals (p=0.3071). However, A71623-treated ($t_{(12)}$=3.361, p=0.0057, n=13) but not saline-treated ($t_{(10)}$=1.102, p=0.2962, n=11) mice were able to perform this task above chance. The exploration time data showed no difference in exploration between the two groups (p=0.3945). This data demonstrates that only mice treated with A71623 were able to successfully perform this task.

As both groups pre-treatment were able to perform all component parts of the OPC task above chance further exploration of the data was undertaken. As all mice explored similarly and the length of time exploring does not seem to differ greatly from pre-treatment tests effect of drug or injection on activity levels were ruled out as being cause for under performance in the OPC task. As such further analysis looked at the discrimination index results only. First performance on the OPC task by day and treatment was looked at to try and identify if performance worsened or improved with practise (**Figure 44**).

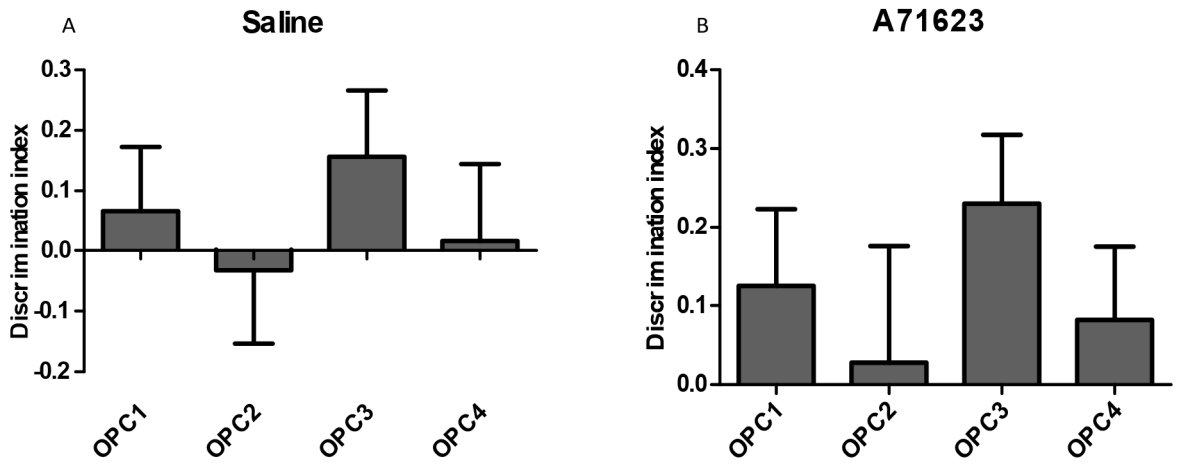

Figure 44. OPC discrimination index scores for Saline and A71623 treated mice by day. Graphs show Mean + SEM discrimination index scores for Saline (A; $F_{(3,38)}$=0.5034, p=0.6822, n=11) and A71623 (B; $F_{(3,43)}$=0.6603, p=0.5810, n=13) treated mice on each day of the OPC task. OPC1 = day 1, OPC2 = day 2, OPC3 = day3 and OPC4 = day 4.

Whilst no significant differences in seen in either treatment group across days (saline p=0.6822, A71623 p=0.5810) there is a trend in both treatment groups towards poorer performance on OPC2 and OPC4. As such an investigation of events on these two days is undertaken separately beginning with OPC2 (Figure 45).

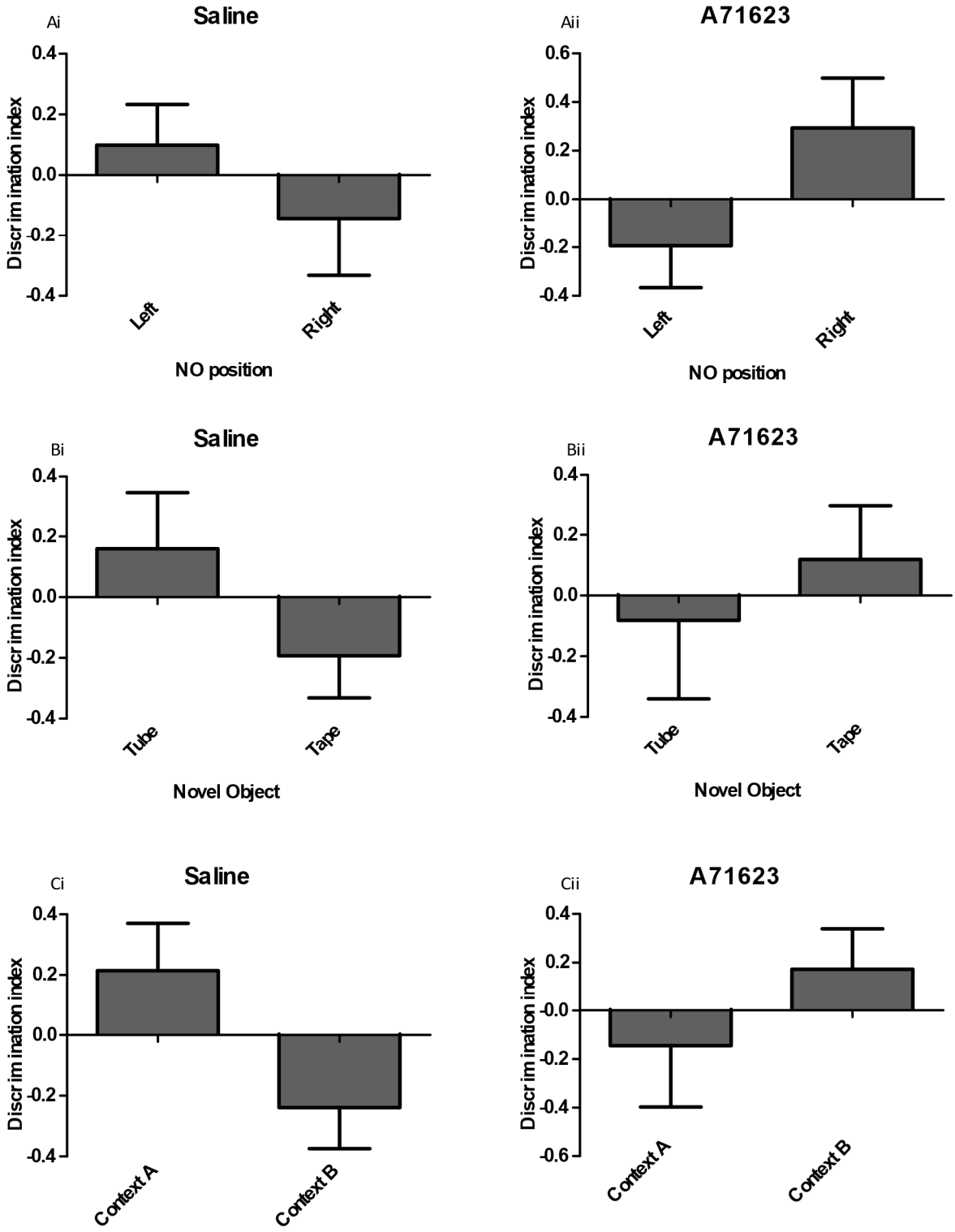

Figure 45. Performance on the OPC task on day 2 shows no common factor effecting both treatment groups. All graphs show mean discrimination index +SEM. A - shows results for saline-treated ($t_{(9)}$=0.9985, p=0.3441, n=6) (i) and A71623-treated ($t_{(9)}$=1.835, p=0.0997, n=6) (ii) mice when the position of the novel object was on the left or right. B – Shows the results for saline-treated ($t_{(9)}$=1.557, p=0.1538, n=6) (i) and A71623-treated ($t_{(9)}$=0.6597, p=0.5260, n=6) (ii) mice when the novel object was the Tube or the Tape. C – Shows the results for saline-treated ($t_{(9)}$=2.201, p=0.0553, n=6) (i) and

A71623-treated ($t_{(9)}$=1.082, p=0.3073, n=6) (ii) mice when the novel object was in context A or context B.

No significant differences were found in any of the unpaired t-test's performed to determine if there was an impact on performance from any of the individual factors of the OPC task, namely the position of the novel object (saline p=0.3441; A71623 p=0.0997) , the nature of the novel object (tube or tape dispenser: saline p=0.1538; A71623 p=0.5260) or the context in which the novel object was placed (A or B: saline p=0.0555; A71623 p=0.3073). However, saline treated mice demonstrated a trend towards poorer performance when the novel object was on the right, was the tape or was in context B and A71623 treated mice seemed to have the exact opposite trend where poorer discrimination was seen when the novel object was on the left, was the tube or was in context A. Looking at the relationships between these events no obvious bias towards any combinations of these factors was clear in either treatment group. The saline treated mice were exposed to the tape being the novel object on the right 3/6 trials, the tape in context B 3/6 trials and the novel object on the right in context B 3/6 trials. In total they saw the combination of the novel object being the tape, with the novel position being on the right in context B 2/6 trials. For the A71623 mice a slight bias does occur as one animal who explored less than 5 seconds occurs in every combination of these factors so once removed the total number of animals becomes out of 5 rather than 6. A71623 treated mice saw the novel combination of the tube on the left 3/5 trials, the tube being novel in context A 3/5 trials and the novel object on the left in context A 3/5 trails. The combination of the novel object being the tube in context A positioned on the left was experienced in 2/5 trials. Looking at whether time of day affected these scores for each of the three-way combinations one occurred in the early morning and one in the late morning or afternoon. As such no obvious lapses in protocol appear to be responsible for poor performance during OPC2.

As performance was also lower on OPC4, though not as markedly as OPC2, the data from this day was also examined more closely (Figure 46).

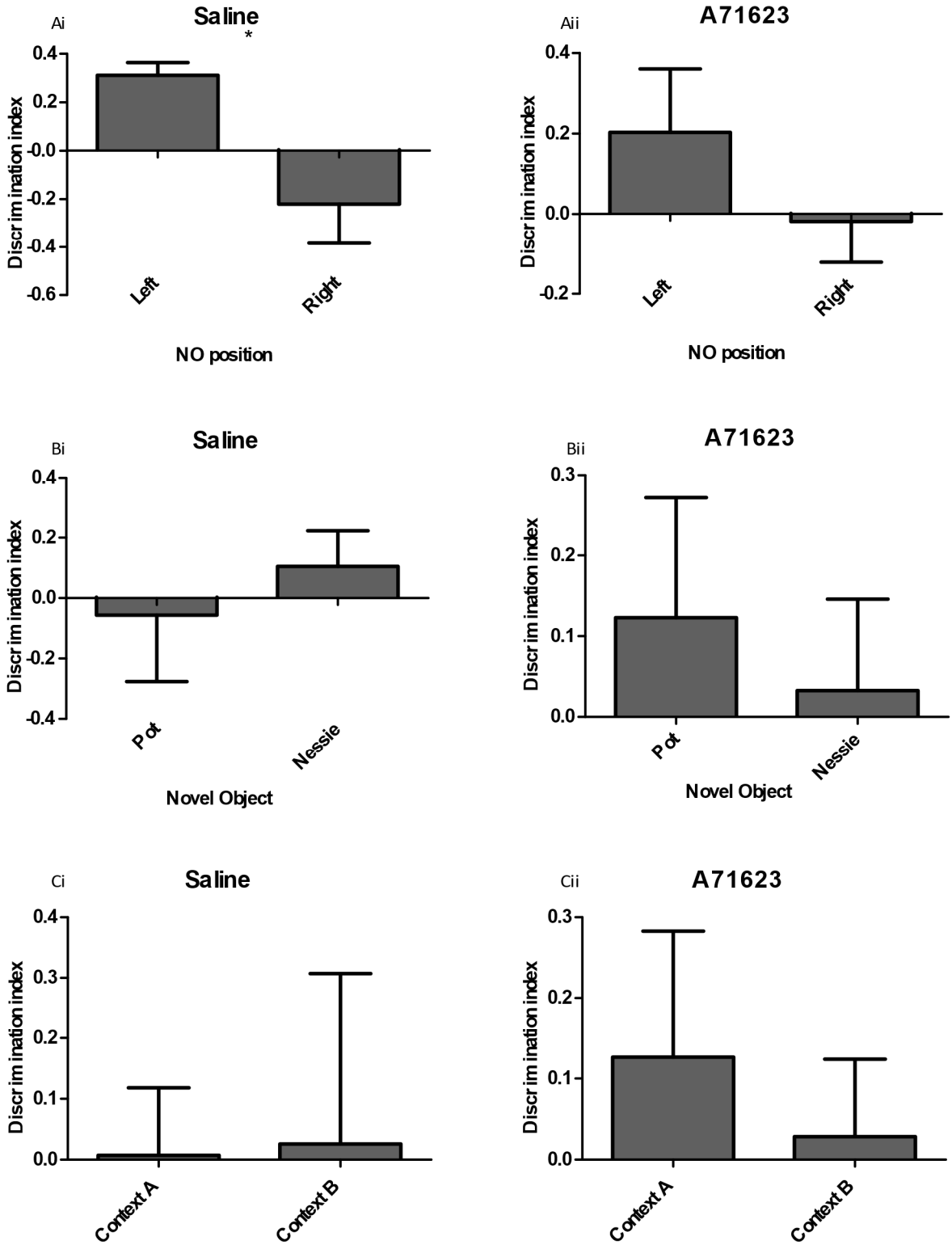

Figure 46. Performance on the OPC task on day 4 shows poorer performance when the novel object is on the right. All graphs show mean discrimination index +SEM. A - shows results for saline-treated ($t_{(7)}$=2.791, p=0.0269, n=5) (i) and A71623-treated ($t_{(9)}$=1.22, p=0.2535, n=6) (ii) mice when the position of the novel object was on the left or right. B – Shows the results for saline-treated ($t_{(7)}$=0.5957, p=0.5701, n=5) (i) and A71623-treated ($t_{(9)}$=0.4652, p=0.6528, n=6) (ii) mice when the novel object was the Pot or Nessie. C – Shows the results for saline-treated ($t_{(7)}$=0.06632, p=0.9490, n=5) (i) and

A71623-treated ($t_{(9)}$=0.5062, p=0.6249, n=6) (ii) mice when the novel object was in context A or context B.

The novel objects in OPC4 were a pot and a Loch Ness monster snow globe (Nessie). When the novel object was positioned on the right the d.i. for the saline treated mice was significantly worse than when it was on the left (p=0.0269). However, no other significant differences were seen for novel object position (left vs right: A71623 p=0.2535), novel object type (pot vs Nessie: saline p=0.5701; A71623 p=0.6528), or context novel object was seen in (A vs B: saline p=0.9490; A71623 p=0.6249). A71623-treated mice also showed a slight trend towards lower discrimination index scores when the object was on the right, however.

Finally, consideration was given to sample phases and alternative patterns of results which may explain the data. The component parts of the OPC task (object place and context) are encoded during the two sample phases. Thus a lack of exploration during either of these two phases will lead to poor performance in the test phase. Alternatively, subtle variations in experimental protocol can lead to mice encoding a different pattern in the object recognition. A possible alternative paradigm is the object-place-temporal order task (OPT) whereby the animals are recognising the object they have seen least recently in a specific position without encoding the contextual nature of the task (Barker *et al.*, 2007)

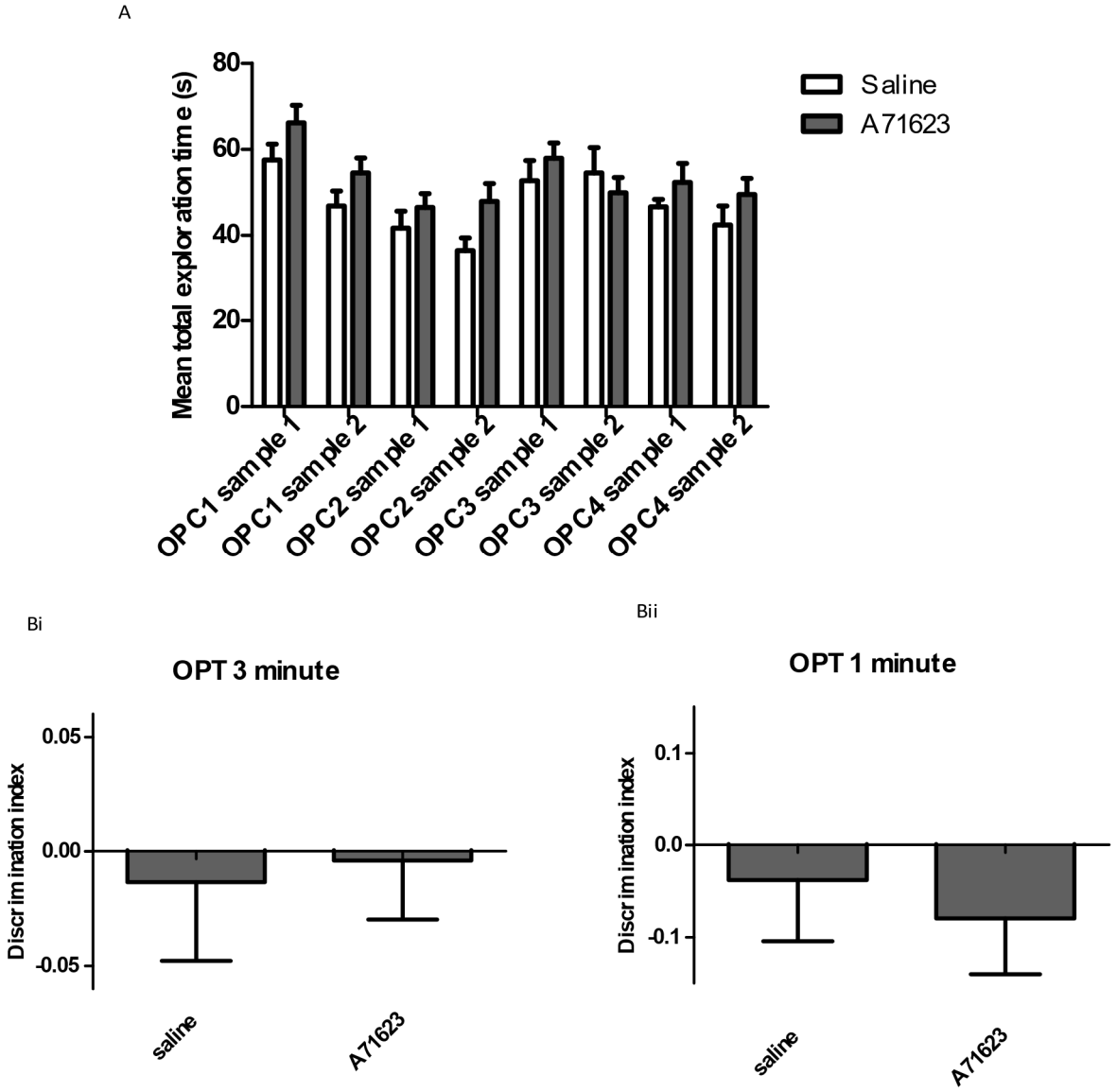

Figure 47. Mice demonstrated robust exploration in all sample phases and did not perform the OPT task. Graphs show mean + SEM. A- The mean exploration times or saline and A71623 treatment mice for each sample phase on each day of the OPC task. There was no significant difference in exploration times between saline and A71623 treated mice in any sample phase on any day (Treatment: $F_{(1,161)}$=2.010, p=0.1697). B – shows the discrimination index for the OPT task at 3 minutes (i) and 1 minute (ii). There was no significant difference between groups for either time point (3minutes: $t_{(23)}$=0.2215, p=0.8266; 1minute: $t_{(23)}$=0.4645, p=0.6467) and neither group performed significantly better than chance (3minutes: saline $t_{(11)}$=0.3917, p=0.7028, A71623 $t_{(12)}$=0.1566, p=0.8781; 1minute: saline $t_{(11)}$=0.5765, p=0.5759, A71623 $t_{(12)}$=0.1321, p=0.2111).

Exploration time in the sample phase varied across days ($F_{(7,161)}$=10.08, p<0.0001) however it did not vary with treatment ($F_{(1,161)}$=2.010, p=0.1697) and treatment did not influence exploration across days ($F_{(7,161)}$=1.365, p=0.2237). All mice explored in the sample phases longer than the minimum 5 second requirement, as such it is unexpected that a problem with encoding occurred. Further,

exploration data was reanalysed to consider the object least recently seen in a position in the test phase to be the novel object to assess if mice were performing the OPT task. Discrimination indexes at both the full 3-minute and 1-minute time points indicate negative average scores for both treatment groups. There was no significant differences between discrimination indexes of saline or A71623 treated mice (3minutes: $t_{(23)}$=0.2215, p=0.8266; 1minute: $t_{(23)}$=0.4645, p=0.6467) and neither group performed above chance levels in this task (3minutes: saline $t_{(11)}$=0.3917, p=0.7028, A71623 $t_{(12)}$=0.1566, p=0.8781; 1minute: saline $t_{(11)}$=0.5765, p=0.5759, A71623 $t_{(12)}$=0.1321, p=0.2111). Therefore, the mice were not performing the OPT task.

6.11.3 A71623 had no significant impact on mouse weight over the course of testing

As activation of the CCK1R has satiety-related functions it was important to assess whether agonism of the receptor by A71623 treatment had an impact on the weight of the mice over the course of the memory experiment. As such mice were weighed twice during the experiment, once before first injections during OPC1 and once after completing the memory task on OPC4 (**Figure 48**).

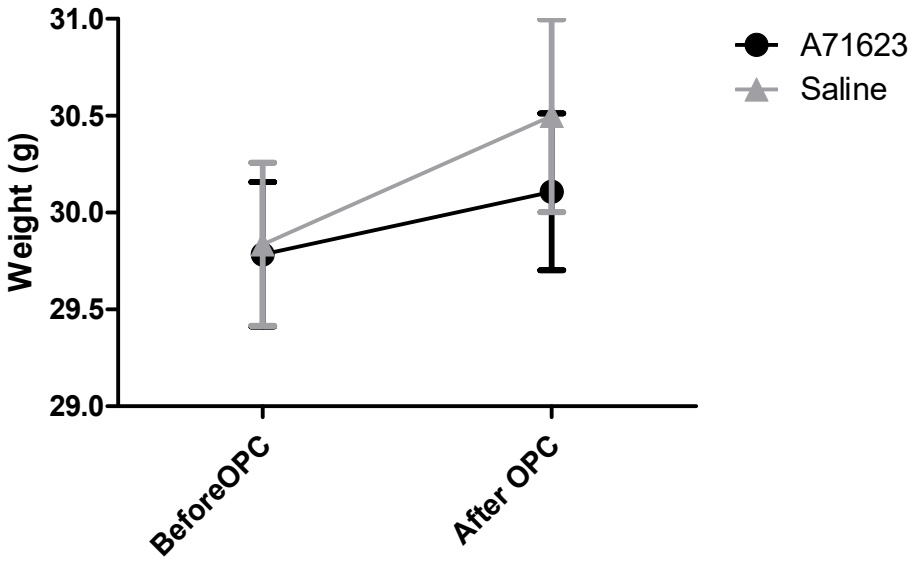

Figure 48. There was no significant change in mouse weight over the course of injections with either treatment. The line graph shows mean ± SEM weight for saline and A71623 treated mice before the first day of OPC task and after completing the final day of testing (Treatment: $F_{(1,22)}$=0.1502, p=0.7021; Time: $F_{(1,22)}$=8.124, p=0.0093; Subjects (matching): $F_{(22,22)}$=10.95, p<0.0001; Interaction: $F_{(1,22)}$=0.9678, p=0.3359; n=13) .

A significant overall effect of time ($p<0.01$) and subjects matched weights ($p<0.001$) was found but no significant difference between saline and A71623 treated mice before or after OPC testing was seen ($p>0.05$ for both). There was no difference in weight therefore, of mice in the A71623 treated group compared to the saline group after 4 days of injections.

6.11.4 Protein extracted from brains of mice after OPC memory task did not show any changes in CCK1R expression by ELISA

Following completion of the OPC task mice were immediately culled by cervical dislocation in accordance with Schedule 1 of UK Animals (Scientific Procedures) Act, 1986 and their brains extracted for assessment of CCK1R expression by ELISA (**Figure 49**). Protein was extracted from one hemisphere for each mouse and CCK1R expression detected using the in-house ELISA method (see **Table 3**).

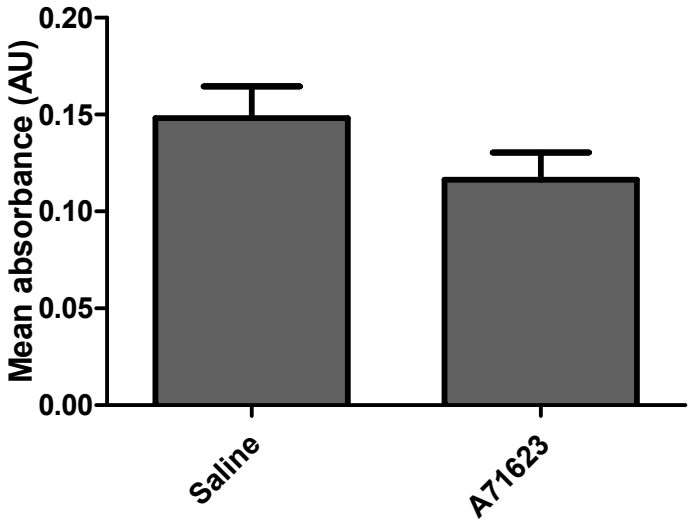

Figure 49. Treatment with A71623 for four days had no significant effect on the expression of CCK1R in the brains of mice. The graph shows the mean + SEM absorbance, as determined by ELISA, of CCK1R expression in whole brain extracts from mice after completing the OPC memory task treated with either saline or A71623 ($t_{(23)}=1.49$, $p=0.1498$, $n=13$).

No significant difference in CCK1R expression in the brains of mice treated with saline or A71623 was seen ($p=0.1498$). This shows there was no significant change in CCK1R expression in the brains of mice treated with A71623 for four days.

6.12 Discussion

This chapter aimed to identify if agonism of the CCK1R could have protective effects in the SHSY-5Y cell line. The specific agonist A71623 was used to determine initially if protection could be seen in undifferentiated cells from induced apoptosis and necrosis. Further assessment of these protective effects were explored by looking at A71623's abilities to modulate NS and OS. After establishing protective effects, I explored if CCK1R agonism could act as a neuroprotective in AD by using disease relevant scenarios. Signalling mechanisms and modulation of AD biomarkers were investigated, and finally I addressed the role of CCK1R as a cognitive enhancer.

Previous studies have demonstrated the presence of CCK1R in differentiated SHSY-5Y cells (Wen *et al.*, 2012) however no clear evidence can be found that expression of this receptor has been proven in the undifferentiated cell line. As such this study showed definitively, using ICC and western blotting, that CCK1R is present and widely expressed in the undifferentiated SHSY-5Y model. This was important to establish so that I could be certain the target of the drug was present and available in this model.

After identifying the presence of the CCK1R, and hence being certain A71623 would have a site of action, exploration of its protective capabilities could be undertaken, using SGD to induce apoptosis and heatshock to induce necrosis. From the SGD data I found 2nM A71623 increased mitochondrial activity and both 2 and 10nM A71623 demonstrated reduced LDH release compared to SGD treatment alone. Further, images of 2nM A71623 treated cells show greater cell number, confirming A71623 was able to protect from SGD-induced apoptosis. This concurs with previous studies which suggest inhibition of CCK1R is linked with apoptosis (Carrillo *et al.*, 2009) and moreover suggests the abilities of CCK itself to rescue from cell death (Lavine *et al.*, 2010) are modulated through CCK1R. This is further supported by the heatshock data, which shows 2 and 10nM A71623 treatments increase mitochondrial activity and 2, 10 and 50nM lower LDH release relative to heatshock alone. Images from cells treated with heatshock or heatshock and 2nM A71623 show greater cell number with A71623 treatment,

confirming the results of the bioassays, thus showing CCK1R agonism can also protect from necrosis. From these results it was determined A71623 was an effective protective agent and further 2nM had consistent action across all conditions. As such this concentration was used to further explore its protective mechanisms.

Both nitrosative and oxidative stress have been linked to apoptotic (Banerjee *et al.*, 2014; Weng *et al.*, 2016) and necrotic (Hanus *et al.*, 2013; Islam *et al.*, 2015) cell death. As such this study explored if CCK1R agonism through A71623 was modulating these events in order to produce its protective effects under SGD and heatshock conditions. First NO activity was examined in the SGD condition. Images taken from DAF-2DA stained cells revealed SGD increased NO production relative to the control condition and 2nM A71623 and 10μM SMTC alone were able to reduce this increase, although not to control levels. When images were analysed for corrected total cell fluorescence a similar pattern in intensity emerged and whilst an overall significant effect could be found, post-hoc testing could not identify where this difference lay. However, a t-test between SGD and 2nM A71623 reveals a significant decrease in NO with CCK1R agonism ($p=0.0111$) as such trends in this data merit discussion. Neither L-NIO (eNOS inhibitor) nor SMUT (iNOS inhibitor) showed trends towards decreasing NO in these cells under SGD conditions suggesting these were not involved in its mechanism of action. SMTC is an nNOS inhibitor as such these results suggest the SGD condition is upregulating NO production through nNOS, as inhibiting this decreases NO in SGD treated cells ($p=0.1292$). Previous links have been identified between SGD and nNOS in the SK-N-BE human neuroblastoma line whereby overexpressing nNOS prior to SGD produced a protective effect (Ciani *et al.*, 2002) it is possible this is due to an acclimatisation effect whereby pre-treatment allowed cells to adapt to SGD conditions. This idea is supported by work in SHSY-5Y cells where pre-treatment of cells with non-lethal SGD increased the expression of nNOS and NO production which reduced the effectiveness of lethal SGD treatment. It was hypothesized the NO-cGMP-PKG signalling pathway is key to this preconditioning effect (Andoh, Chock and Chiueh, 2002). CCK has been shown to activate the NO-cGMP-PKG pathway in CHO cells expressing CCK1R leading to increased NOS activity and NO production (Dufresne, Seva and Fourmy, 2006) which may

explain why in combination A71623 and SMTC demonstrate increased NO. Further cGMP has been shown to increase interleukin-1 induced NO production in smooth muscle by upregulating iNOS (Inoue *et al.*, 1995) and as CCK itself can directly increase cGMP (Silvente-Poirot *et al.*, 1993) it is possible that in the combined A71623 and SMTC condition this mechanism is being activated resulting in the increased NO. However, this does not explain how A71623 alone is decreasing NO. In a study looking at the relationship between CCK and NO animals were treated with nNOS inhibitor with or without CCK-8, it was found that when CCK-8 was given in combination with nNOS inhibitors the effects produced by the nNOS inhibitor alone disappeared. This suggests a functional co-operation between CCK and NO (Ferraro *et al.*, 2003). Given the Janus nature of NO (Calabrese *et al.*, 2009) it is possible that CCK produces a modulating effect such that under conditions of stress, such as SGD, where NO production is increased CCK through the activation of CCK1R is able to decrease NO production. Conversely, in the scenario where NO production is being supressed e.g. through nNOS inhibition, CCK acts to increase NO levels.

From the LDH data A71623, SMUT and SMTC alone as well as A71623 with L-NIO or SMUT had significantly reduced LDH release relative to the SGD control, suggesting improved cell viability. I have already demonstrated that A71623 can protect against SGD induced cell death, so this result is consistent with this. Data from the eNOS inhibitor L-NIO alone does not show a protective effect in this assay. As eNOS activates the PI3K-Akt pathway and has therefore been linked to protective cell-survival effects in many tissues (B. Zhang *et al.*, 2016; Chu *et al.*, 2017; Hu *et al.*, 2017; Zhou *et al.*, 2018), it follows then that inhibiting eNOS would prevent these protective effects as our data shows. However, co-treatment with A71623 is able to produce a protective effect therefore indicating it is working through a different pathway. The data for the iNOS inhibitor SMUT shows that both alone and in co-treatment with A71623 there is a protective effect. In renal ischemia-reperfusion injury rats iNOS, NO, cGMP and PKG are all increased and lead to apoptosis of renal tubules. Suppressing this pathway protected against cell death (Liu *et al.*, 2017). Further, in a modelled immune response CCK was found to reduce NO and iNOS production and these actions were via CCK1R (Saia *et al.*, 2014). As such the

results from iNOS inhibition by SMUT with and without A71623 are consistent with this previous work. Finally, in a pattern reminiscent of the DAF2-DA staining, the nNOS inhibitor SMTC shows protective abilities on its own but in co-treatment with A71623 this effect disappears. This is supportive of the earlier theory that CCK can modulate NO levels under different conditions and thus what may be happening in the combined A71623 and nNOS inhibitor treatment group is an overcompensation for the reduction in NO which results in toxicity.

NO activity was also examined after cells had undergone heatshock. Images taken from heatshocked cells stained with the NO-sensitive DAF2-DA dye suggest that heatshock alone did not increase NO production. This may be supported by previous work that suggests heatshock can inhibit the interleukin-1β induced iNOS expression, as seen in rat and human islets (Scarim, Heitmeier and Corbett, 1998). Whilst little variation in the images is otherwise seen the data from the corrected total cell fluorescence measures demonstrates similar patterns, although this data did not reach significance. From the graph both conditions containing the iNOS inhibitor SMUT also demonstrate lower detection of NO under heatshock conditions which may support the hypothesis. However, A71623, eNOS inhibitor and nNOS inhibitor all indicate higher NO levels despite heatshock itself not increasing NO production. As previously mentioned CCK can activate the NO-cGMP-PKG signalling pathway to increase NOS activity and NO production, as such it may be under heatshock conditions activation of the CCK1R increases NO. This could be linked to an immune response. Heatshock and the release of heatshock proteins has been linked to the immune response (Zheng *et al.*, 2003; Guisasola *et al.*, 2018) as such it may be that CCK1R activation is increasing NO as part of this response. CCK has previously been linked to the immune response. In rats with acute pancreatitis inhibiting CCK through the antagonist L364,718, selective for CCK1R, prevented early immune events (de la Mano *et al.*, 2004). Further, peripheral blood monocytes treated with CCK showed increased proinflammatory mediators and neutrophil chemotaxins compared to untreated control cells, suggesting CCK may up-regulate gut-immune response (Cunningham *et al.*, 1995). However, little work has currently been done to link these theories together.

Heatshock has been linked to eNOS through the eNOS-Akt-HSP90 pathway. Research has shown HSP90 increases eNOS activity synergistically with Akt (Takahashi and Mendelsohn, 2003) hence I would expect inhibiting eNOS would not produce increased NO in these cultures. However, other studies have also demonstrated that inhibiting eNOS by L-NIO increased iNOS, and therefore NO, in an immune response (Greco *et al.*, 2018).

Finally, I examined whether A71623 could itself modulate any of the NOS isoforms. As such after 24h treatment with 2nM A71623 or under control, untreated conditions protein from undifferentiated SHSY-5Y cells was extracted and ELISAs for nNOS, iNOS and eNOS carried out. The data showed that A71623 treatment alone had no effect on nNOS or eNOS levels. However, treatment significantly reduced iNOS levels, which is in accordance with previous research, which shows suppression of iNOS protected against cell death (Liu *et al.*, 2017) and CCK reduced iNOS in a modelled immune response via CCK1R (Saia *et al.*, 2014).

Oxidative stress has also been linked to apoptosis and necrosis. To explore whether A71623's protective effects were enacted through modulation of OS under SGD and heatshock conditions superoxide levels were measured. Under SGD, which initiates an apoptotic death pathway, cells showed increased superoxide production relative to the 2nM A71623 under control conditions. However, A71623 treatment could not prevent superoxide increase under SGD conditions. Previous studies have shown that serum deprivation decreases SOD activity (Seo *et al.*, 2013), using a superoxide anion scavenger under low serum conditions protects cells from apoptosis (Nishina *et al.*, 2011) and in serum deprived conditions addition of polyethylene glycol conjugated SOD rescued cell cultures (Lieven *et al.*, 2012), which support the notion that SGD increases superoxide activity. Little work has examined the relationship between superoxide and CCK specifically. However, one study looking at human neutrophils found no changes in superoxide anion production in the presence of CCK-8 (Carrasco *et al.*, 1997) but 10nM CCK-8s reduced superoxide production in murine macrophages (De la Fuente *et al.*,

1995). It is possible, therefore, that CCK's effects on superoxide are tissue or species dependent but may also be controlled by CCK2R as opposed to CCK1R hence no modulation was evident in this study.

Under heatshock conditions, there was no increase in superoxide and 2nM A71623 had no other modulatory effects. Previous work suggests that heatshock may increase SOD activity (Yamashita *et al.*, 1997; Yoo, Chang and Rho, 1999), as such it is not surprising that no increase in superoxide was seen in this experiment. Further the link between heatshock and OS more generally gives rise to debate. Evidence from cells heatshocked in anaerobic environments demonstrate greater resistance than those in an aerobic environment, additionally, cells lacking antioxidant enzymes are more sensitive to heat shock (Davidson *et al.*, 1996) suggesting oxygen availability and oxidative stress are an important function of heatshock. However, heatshock also upregulates the antioxidant glutathione (Sugiyama, Izawa and Inoue, 2000) and HSPs have been shown to protect against oxidative stress (Yurinskaya *et al.*, 2015; Donovan, Marr and II, 2016; Alvarez-Olmedo *et al.*, 2017). Due to this conflicting evidence exploration of the effects of heatshock and A71623 with regards to oxidative stress was not pursued further.

As SGD condition could increase superoxide but A71623 could not protect from this specific increase in oxidative species a more general method of oxidative stress detection was employed to assess A71623's abilities. It was seen that SGD drastically increased OS and A71623 did not protect from this. In agreement with our study, serum deprivation has previously been shown to increase ROS and OS (Pandey, Lopez and Jammu, 2003; Yoo *et al.*, 2011; Mirzamohammadi *et al.*, 2016). One previous study has shown that CCK-8 treatment inhibits methamphetamine neurotoxicity by reducing oxidative stress, however it was concluded that this action was mediated viaCCK2R (Wen *et al.*, 2016).

In combination the data from studying nitrosative and oxidative stress suggests that under SGD conditions CCK1R agonism can reduce NO and may demonstrate some capability to modulate NO production through the activation of the NO-cGMP-PKC pathway. However, it is unable to counteract the large increase is OS in these conditions despite its demonstrated protective ability. Whilst heatshock

alone did not affect NO or superoxide production the data from the A71623 treatments further lend credence to the theory of CCK modulating NO production, whilst having little influence on oxidative stress.

As A71623 was protective in the undifferentiated SHSY-5Y cell line, I explored whether these effects would also be seen in the mouse hippocampal cell line HT22. As previous experimentation demonstrated that serum deprivation induced significant cell death in these cells (see 5.7), this method was used for these experiments. From CV assay it was seen that under 10% serum control conditions 50nM A71623 had reduced cell number compared to controls. This suggests that the highest concentration of A71623 being used in these experiments was toxic to HT22 cells or was causing them to exit from the cell cycle. However, 2nM A71623-treated cultures had a significantly higher cell number than 50nM indicating this concentration was not having these effects. For the serum deprivation conditions, no concentration of A71623 showed a difference from the serum deprivation control in terms of cell number or LDH release. Therefore, A71623 was unable to protect from cell death induced by serum deprivation in HT22 cells. In order to ascertain if CCK1R expression changed with differentiation and therefore A71623 may have different effects post-differentiation, expression levels were examined by ELISA. No differences were found in receptor expression in undifferentiated and differentiated cells. No previous research has been done on CCK in HT22 cells. In the rat pancreas differential strategies of CCK-8 exposure causes cell proliferation and apoptosis. With continuous exposure cell proliferation and apoptosis were evident, however with intermittent CCK-8 exposure only apoptosis was seen. This effect is seen with high doses of CCK and is caspase dependent (Trulsson *et al.*, 2001). This data may explain why 50nM A71623 induced cell loss in the control condition.

As A71623 could protect from induced apoptosis and necrosis in undifferentiated SHSY-5Y cells I wanted to explore its abilities in differentiated SH-SY5Y cells and furthermore to use AD relevant toxic conditions to explore A71623's potential as a neuroprotective agent for AD therapy. First it was

confirmed that the receptor was still expressed post-differentiation and that significant changes did not occur at the stage at which cells were used in experiments as this may affect how cells respond to treatment. The data show the CCK1R is present at all stages of differentiation explored. From the ICC images similar levels of CCK1R is expressed in individual cells and this is confirmed by ELISA which demonstrated no significant change in expression with differentiation.

Once the receptor's presence was confirmed, the efficacy of A71623 as a neuroprotective agent was explored using divalent metal ions that are often used to model neurodegeneration *in vitro* (copper chloride) and an AD specific neurotoxin (Aβ). Data from MTT, LDH and CV assays demonstrate that 2nM A71623 could consistently protect mitochondrial activity, reduce LDH release and increase cell number relative to the copper condition and therefore protect from copper chloride induced cytotoxicity. This was confirmed visually through photomicrographs. Further the data also shows that both 0.4 and 2nM could protect mitochondrial activity, reduce LDH release and protect against cell number decrease in Aβ-treated cells. Previous studies have demonstrated the protective effects of CCK whereby inhibiting CCK1R induced apoptosis in Ewing tumour cells (Carrillo *et al.*, 2009), loss of CCK in C57BL/6 mice induced beta-cell death and addition of CCK in culture could rescue these cells (Lavine *et al.*, 2010). However, this research shows for the first time that direct agonism of CCK1R can induce neuroprotective effects and do so under AD-relevant conditions.

Previous work examining neuropeptide changes in the hippocampus and cortex in the AD mouse models APP 717V->F and APP23 found that in both cases there was decreased CCK in mossy fibres. For the APP 717V->F mice CCK increased in the stratum lacunosum moleculare (Diez *et al.*, 2000) and for the APP23 mice CCK was commonly seen in close proximity to Aβ plaques in hippocampal formation, dorsolateral neocortex and ventral cortex (Diez *et al.*, 2003). Together these papers suggest a reduction in CCK might lead to changes in excitability of the hippocampus with a trend towards inhibition (Diez *et al.*, 2000, 2003). The co-localisation of CCK with Aβ plaques as seen in these mice, in combination with my work showing CCK1R agonism can protect against Aβ toxicity suggest a neuroprotective role within

AD models for CCK which is hampered by its declining levels. However, in a study looking at post-mortem cerebral cortex tissue from AD patients it was seen that CCK was decreased in many areas across the cortex but with no changes in the hippocampus (Mazurek and Beal, 1991). Other post-mortem studies have also found similar effects of decreases in CCK in parts of the cortex and no change in hippocampus but these did not reach significance (Rossor *et al.*, 1981; Ferrier *et al.*, 1983). However, it was found that post-mortem delay had a large impact on these findings with a 70% decrease in CCK found in AD brains with a processing delay of 22hours or less but delays longer than this saw much lower changes in CCK (Perry *et al.*, 1981). Drawing firm conclusions from this work therefore becomes problematic. In comparison, in mouse studies no changes were seen in CCK levels in the hippocampus which could be due to variances in the models or species used, but it may also be due to time post-mortem that samples are measured. Even in the study with clear changes seen in the cortex, post-mortem delay was an average of 13hours (Mazurek and Beal, 1991) whereas in the animal studies the mice were anesthetized and perfused directly (Diez *et al.*, 2000, 2003), resulting in no post-mortem delay. It is possible that changes in the hippocampus are particularly sensitive and cannot be detected in humans at the time point seen. Further, the post-mortem studies do not indicate Braak's stage of AD. It is possible that their patient samples have different stages of AD which may disguise changes in neuropeptides relative to specific stages, or, more likely as they are using post-mortem samples, the brains they were looking at were relatively late-stage AD and as such, due to the brain's degenerative changes, measuring changes in neuropeptides may no longer be reliable.

CCK1R has been shown to signal via the PI3K/Akt pathway (Zhou *et al.*, 2014) and CCK-8 has been shown to activate the STAT3 pathway (Heldsinger *et al.*, 2011), both of which are pro-survival pathways (Orike *et al.*, 2001; Guo *et al.*, 2008; Beales and Ogunwobi, 2009). As such it was investigated whether CCK1R agonism via A71623 could increase activation of STAT3 and/or Akt, showing it is acting through these pathways to enact its neuroprotective effect. A71623 significantly increased

phosphorylation of STAT3 but not Akt indicating it is acting via the STAT3 pathway to regulate these neuroprotective effects. Previous work has shown CCK increased nuclear localisation of pSTAT3 in acinar cells and suggested STAT-SOCS signalling was important in CCK-dependent pancreatic growth (Gurda *et al.*, 2012). CCK-8 has been shown to increase Src kinase and protein tyrosine kinase (PTK) (Tsunoda *et al.*, 1996), as STAT3 contains an Src domain CCK could directly activate it (Heldsinger *et al.*, 2011) and our results would suggest this is via CCK1R activation. However, PI3K also has an Src domain (Nozu, Owyang and Tsunoda, 2000) as such this seems contrary to my result where pAkt, downstream of PI3K, was not increased with A71623 treatment. However, it is possible there is a mechanism through which PI3K activates STAT3 without the involvement of Akt. One such pathway is the PI3K/BMX/STAT3 pathway which has been implicated as a therapeutic target in cancer (Vogt and Hart, 2011). In small cell lung cancer reduced phosphorylation of the PI3K/BMX/STAT3 signalling pathway was linked with increased apoptosis and therefore decreased chemoresistance (Peng *et al.*, 2016). As such, it is possible that under healthy conditions activation of this pathway prevents apoptosis and would be a cell survival pathway, which would fit with my findings. Further exploration of these pathways is required to elucidate these effects however, for example by using inhibitors specific to different molecules in the pathway and determining how this impacts the neuroprotective abilities of A71623.

As with leptin and leptin$_{116\text{-}130}$ it was important to assess the effects of A71623 treatment on biomarkers in an AD empirical model to understand if it could modulate disease progression. Similar to the previous experiment (see 5.5) consistently producing cultures which met the criteria of use (increased p-tau in Aβ treatment condition relative to control) was difficult. However, in this set of experiments one biomarker was able to produce a significant effect. It is thought that this is due to the higher number of experimental repeats which were successful for this biomarker. The remaining biomarkers had lower numbers experimental repeats as such this may be the cause for lack of significance. As such the trends in the data may be worth assessment.

The one biomarker which reached significance in this set of experiments was p-tau. My results demonstrate that 1μM Aβ significantly increases phosphorylation of tau at ser262 as described previously (Manassero *et al.*, 2016; Bennett *et al.*, 2017). Further, 2nM A71623 co-treatment with 1μM Aβ significantly reduced p-tau levels indicating it can successfully reduce this biomarker. In OLETF rats, which lack the CCK1R, increased phosphorylation of tau at a number of sites, including ser262, is seen with age (Jung *et al.*, 2013), which lends support to my data. No significant changes in tau levels could be seen with these samples, which is the only other biomarker to be tested on the same number of experimental repeats. This demonstrates that neither 1μM Aβ nor 2nM A71623 had an effect on tau itself *per se*. Whilst little work has been done on the effects of CCK on tau a recent study investigated levels of CCK in healthy, MCI and AD patients in relation to CSF levels of tau and phosphorylated tau. Regardless of diagnosis higher CSF levels of CCK were significantly correlated with higher levels of both total tau and p-tau. However higher levels of CCK were also significantly correlated with better cognition and memory scores; grey matter volume in cingulate cortex, parahippocampal gyrus, thalamus, superior temporal sulcus and medial prefrontal cortex; and decreased likelihood of having MCI or AD, or converting from MCI to AD. Investigating, therefore, how tau and p-tau levels impacted CCK's influence on memory scores it was seen that on the ADAS-cog-11 higher total tau reduced the influence of CCK and on the MMSE higher p-tau reduced the influence of CCK. Further looking at specific diagnosis it was seen that for MCI patient's total tau reduced the influence of CCK further but for cognitively normal and AD patients there was no additional mediation effect (Plagman *et al.*, 2019). This study would suggest that rather than causing changes in tau itself, CCK *in vivo* is acting as a protectant, increasing as tau levels and phosphorylation do in order to combat pathology but reaching a point at which increases in tau species overcome protective effects of CCK. My data would suggest that acting through the CCK1R, CCK can modulate p-tau but has little effect on tau. This may suggest that in the early stages of tau pathology CCK1R activation enacts its protective role by modulating phosphorylation of tau but that this mechanism can be overcome as pathology builds.

The ABAD ELISA may suggest a subtle decrease between Aβ treatment and Aβ with 2nM A71623 treatment this is further supported by a t-test between these conditions (p=0.0679). ABAD has been linked to a protective response to metabolic stress, whereby increased ketones resulting from fasting or starvation are utilized by ABAD to produce energy for the brain (Du Yan *et al.*, 2000). CCK release and ketones have previously been suggested to be linked (Chearskul *et al.*, 2008) but as yet no direct testing of this has been carried out. It is possible that the signalling through the CCK1R in our cultures, coupled with plentiful energy source in the cell medium indicates a lack of starvation state and hence a downregulation of ABAD in response. Further repetition of these experiments to attain statistical power is therefore important for future work.

Although I would expect APP to increase in my cultures due to Aβ increasing OS leading to increased expression (Tamagno *et al.*, 2007; Zhao *et al.*, 2007; Picone *et al.*, 2015), I saw no obvious changes in APP levels with 1μM Aβ or 2nM A71623, alone or in co-treatment. Previous work has suggested that feeding hormones impact APP levels in aged rats, as calorie restriction increased APP levels and refeeding these animals reduced them to baseline (Banks, Abrass and Hansen, 2016). Whilst this study did not look at CCK specifically it may suggest that feeding hormones would only impact APP at increased levels and not at normal levels as ageing rats fed *ad libitum* saw no changes in APP or feeding hormone levels in the brain (Banks, Abrass and Hansen, 2016). Therefore, as Aβ did not increase APP in our culture's activation of CCK1R may not have had an impact. Whilst a direct link between CCK and APP remains to be explored, an indirect link via GSK-3 may exist. In rat neuroblastoma cells treatment with Aβ increases Ras-ERK signalling, GSK-3 activation, and tau and APP phosphorylation, conversely knocking-down APP inhibits Ras-ERK signalling and GSK-3 activation (Kirouac *et al.*, 2017), which suggests APP is upstream of this pathway. Further, in both SK-N-MC and PC12 cells treatment with GSK-3 decreased cAMP induced CCK gene transcription (Hansen, Rehfeld and Nielsen, 2004). As such it is unlikely that CCK can affect APP levels, rather increased APP can decrease CCK, but activation of its receptors independently of CCK may have little impact as this is downstream from APP's actions.

Aβ has been shown to increase endophilin 1 in a cellular model of the BBB (Liu *et al.*, 2016) and in AD affected neurons (Yu *et al.*, 2018). The results from this experiment agree with previous work as 1μM Aβ treated cells show increased endophilin 1. However, endophilin 1 also appear to be increased with 2nM A71623 treatment and 2nM A71623 with 1μM Aβ co-treatment. CCK has been shown to increase AMPA receptor-mediated excitatory postsynaptic potentials, increase probability of glutamate vesicle release and number of readily releasable vesicles in hippocampal slices, however this effect was seen to be through CCK2R not CCK1R (Deng *et al.*, 2010). In rat pancreatic acinar cells, CCK stimulated amylase release and this was dependent on SNAP-23 an important component in vesicle transport machinery (Huang *et al.*, 2001), though no suggestion of receptor specificity was made. SNAP-25 is a homolog of SNAP-23 which is primarily expressed in the brain (Yamamori *et al.*, 2011). Endophilin 1 has been shown to interact with SNAP-25-interacting protein (SNIP) (Yang *et al.*, 2015). SNIP co-localises with SNAP-25 and is responsible for linking it with the cytoskeleton and regulation of vesicle secretion (Chin *et al.*, 2000). Endophilin 1 was shown to regulate the distribution of SNIP and decreasing endophilin 1 caused a loss of SNIP (Yang *et al.*, 2015). As such there may be a mechanism by which CCK is upregulating endophilin 1 and in turn increasing vesicle secretion by effecting release machinery.

Presenilin 1 increase can be caused by the positive feedback loop of Aβ whereby Aβ increases OS, which in turn activates JNK and increases expression of presenilin 1 and BACE 1, leading to the increased production of Aβ (Tamagno *et al.*, 2008). My data suggests there is little change in presenilin 1 levels with 1μM Aβ or A71623 treatments. Previous research has seen that there was some co-localisation between filamin, an actin cross-linking protein that interacts with presenilin 1, and CCK in cells containing the familial AD presenilin 1 mutation, however the nature of any relationship is unclear (Lu *et al.*, 2010).

Altogether the AD biomarker data from subtoxic doses of Aβ suggest that CCK1R agonism by A71623 can ameliorate increased p-tau levels and downregulate ABAD (though this may be a nutritional

rather than pathologic response). While much work is still needed on the interactions of CCK and AD biomarkers, this initial data suggests promise for disease modifying effects.

Having demonstrated that CCK1R agonism has neuroprotective effects in an AD model and given earlier data that suggest a role for CCK1R in memory (Lemaire *et al.*, 1992, 1994; Berthelot *et al.*, 1996; Nomoto *et al.*, 1999; Xue-Liang Li *et al.*, 2002; Hadjiivanova, Belcheva and Belcheva, 2003; Matsushita *et al.*, 2003; Ohinata *et al.*, 2007) the ability of A71623 to effect episodic-like memory were tested on the OPC task. In the pre-treatment testing of the component parts of the OPC task no significant differences were found between the two groups of mice. Both groups showed positive discrimination indexes for the novel object above chance levels and demonstrated equal total exploration of objects. However, in the OPC task no significant difference was seen between the discrimination index of saline and A71623 treated mice and neither group were able to perform the task above chance levels as calculated from the 3-minute data. Both groups still explored similarly, and exploration times were similar to pre-treatment levels as such these results are not likely to be due to stress or poor exploration. As previous research suggests interest in the novel object is highest at the beginning of the test and significantly declines thereafter (Clark, Zola and Squire, 2000), data was also analysed from the first minute of testing. Nevertheless, there was no significant difference in discrimination index score between the saline and A71623 group, but the A71623-treated group were now seen to perform the task above chance levels. As exploration times were still not significantly different, this may suggest that the A71623 group is performing better than the saline control as they were able to complete the task above chance levels. However, as the control group, which represents baseline performance, were not able to perform the task it is difficult to draw firm conclusions from this.

Mice can spontaneously perform this task (see 5.6.2) therefore, an attempt to explore any unintended bias in experimental method was undertaken. Both saline and A71623 treatment groups

had lower mean discrimination index scores on days 2 and 4 and so this data was examined more closely. On day 2, the two treatment groups had poorer performance in the exact opposite conditions. The saline treated group had on average negative discrimination indexes when the object was on the right, the tape dispenser and in context B. However, the A71623 treated group had negative average discrimination indexes when the object was on the left, was the tube or in context A. As such no clear trend for object position, object type or context has arisen from this data. One further idea was that these combinations were occurring at a specific time of day. This may indicate a specific event had occurred which affected testing. However, for both saline and A71623 treated mice that experience the 3-way combination of these events they were spread across the testing day. As such no satisfactory methodological or external event-related explanation could be identified for this occurrence.

On exploration of the day four data both treatment groups had a negative average discrimination index when the object was on the right, with this difference being significant in the saline group. Data for object type and context showed opposite trends in preference between the treatment groups and with more variation. In this case it seemed that when the novel object was positioned on the right on this day mice were less prone to exploring it. Whilst the experimenter has no precise knowledge of why this is the case on the fourth day it is likely that a small adjustment could have been made to the experimental set up to cause this effect. In these experiments the experimental set-up was lit by two free-standing lamps which had been positioned to completely light the experimental box without creating shadows in which the mice would prefer to be. The position of these lamps was marked on the floor so if they were moved, they could be returned to the original position. There is a chance that one lamp was moved slightly unknown to the experimenter that created a slight shade in the experimental set-up by the left side object which the animals may have preferred. As this cannot be verified, though, this suggestion is purely speculative.

Given that CCK is a satiety inducing hormone and rats treated with A71623 have demonstrated lower weight than controls (Asin, Bednarz, Nikkel, Gore, Montana, *et al.*, 1992) mice were weighed

before the first day of testing and after completing the memory task on day 4. Overall the mice gained weight over the experiment and there were no significant differences between A71623 and saline treated mice after 4 days of injections. As such this time period of A71623 treatment did not impact the weight of our mice.

To assess any changes in receptor expression the brains of the mice were extracted immediately after each had completed the final OPC task and CCK1R expression levels of whole hemisphere protein extracts measured. No significant changes in receptor expression occurred between saline treated and A71623 treated mice. Research into active immunization against CCK-8 in pigs has shown that giving pigs 250μg but not 500μg CCK-8 per day decreased CCK receptor gene expression. However, this experiment was carried out over 75 days , as such it is less likely I would detect similar results in the mice (Zhang *et al.*, 2007).

Overall, this chapter has demonstrated that the CCK1R is expressed in SHSY-5Y cells both pre and post differentiation and agonism of this receptor has protective effects both on undifferentiated cells and fully differentiated neurons. The data has suggested that A71623 can modulate NO under stress conditions seen to be through its ability to decrease iNOS expression but has no role in modulating OS and could not protect the hippocampal HT22 cells from serum deprivation. Differentiation of the SHSY-5Y cells into a neuronal phenotype demonstrated A71623 was neuroprotective against copper chloride cytotoxicity and AD specific Aβ toxicity, and further these actions were likely to be via the STAT3 cell survival pathway. A71623 was also significantly able to modulate the AD biomarker p-tau, though further work and method refinement is required to understand its relationship with other biomarkers. Whilst A71623 treated mice were able to perform an episodic-like memory task above chance and control groups were not, firm conclusions about its memory enhancing abilities are difficult to draw and require further investigation. However, over a short treatment period A71623 did not affect animal weight or receptor expression in the brain.

Together this data suggests that agonism of the CCK1R through A71623 has potential as an AD therapeutic as it can protect neurons from AD-toxic environments, modulate NS and decrease p-tau levels. As such further exploration into its memory-enhancing potential and route of action should be undertaken.

7 EXPLORING CCK AND LEPTIN'S SYNERGY AND AGE-RELATED CHANGES WHICH MAY IMPACT THEIR EFFICACY

7.1 INTRODUCTION

Leptin, leptin$_{116-130}$ and agonism of CCK1R by A71623 have each been tested under AD disease relevant conditions and indicated abilities to modify disease course. However, AD is a multifaceted disease for which a single effective long-term treatment is yet to be discovered. As such treating it may require more complicated therapeutics which can drive protection and alter pathological states in multiple ways. Work in other fields has highlighted the benefits of combination therapies with additive or synergistic effects leading to lower side effects (Vilar *et al.*, 2015); increased survival (Moul and Chodak, 2004; Kline *et al.*, 2016) and lower doses required to achieve the same effects (Hatkevich *et al.*, 2014). Previous work has suggested combination therapies may be beneficial in AD. It is possible that some pre-existing approved therapies could show improved effects in combination, such as cholinesterase inhibitors, for which a combination therapy meta-analysis showed improved cognition, behavioural disturbances, activities of daily living and global assessment scores (Matsunaga, Kishi and Iwata, 2015), or new treatments such as octyl gallate and ferulic acid which show improved cognition and decreased Aβ deposits, neuroinflammation and oxidative stress compared to monotherapy in APP/PSN1 mice (Mori *et al.*, 2017). This raises the question if leptin and CCK could be used as co-treatments.

A synergistic relationship between CCK and leptin has been identified in their roles as satiety hormones. This interaction was initially observed by investigating effects on food intake. CCK reduced 30-minute caloric intake independently of leptin in adult mice, but had no effect on total daily calorie intake, whereas leptin had no effect on short-term intake but significantly reduced daily intake. When co-administered there were no effects on 30-minute intake, but total daily intake was significantly reduced compared to leptin alone. As CCK itself had no effect on total daily caloric intake this suggests the relationship between CCK and leptin is synergistic rather than additive (Matson *et al.*, 1997). Further, subthreshold co-treatment of CCK-8 and leptin decreased food intake in 24hr lean fasting mice

in the first hour, but leptin alone had no effect on food intake in the first 3 hours. This effect was blocked by the CCK1R antagonist devazepide but not by CCK2R antagonist L-365,260, demonstrating CCK1R was responsible for this action (Barrachina *et al.*, 1997). This CCK1R-dependency is supported by work showing CCK-8 suppressed plasma leptin increase after i.p. injection in rats and increased cerebrospinal fluid leptin levels, which was blocked by CCK1R antagonist SR-27,897 but not CCK2R antagonist L-365,260 (Cano *et al.*, 2008). Pre-treatment of lean-fasted mice with capsaicin blocked the effects of CCK and leptin combined treatment showing this interaction was likely located in capsaicin-sensitive vagal afferents. Co-treatment also increased Fos-positive cells in the hypothalamic paraventricular nucleus suggesting a role in the central neural pathways underlying this combined action (Barrachina *et al.*, 1997).

Leptin causes meal-size reduction, which is dependent on vagal afferent neurons and this effect is enhanced by CCK (Peters *et al.*, 2005). Within cultured rat nodose ganglia vagal afferents, 73% of neurons activated by leptin were also sensitive to CCK, and both leptin and CCK increased sodium-dependent and inhibited potassium-dependent conductance in these neurons. This demonstrates leptin and CCK signalling converges in vagal afferents (Peters, Ritter and Simasko, 2006). This is further supported by work in the Zucker rat, which is leptin sensitive when lean, and leptin insensitive when obese. CCK-8s injection reduced food intake in the lean but not the obese rat, and the vagal afferent neurons of the obese rats demonstrated reduced CCK signalling. This result was replicated in vagal afferent neurons of obese Sprague Dawley rats, which are also leptin insensitive, whereby reduced early growth factor 1 expression led to reduced sensitivity to CCK and hence reduced inhibition of food intake by CCK (de Lartigue *et al.*, 2012). Finally, evidence suggests this synergistic interaction is mediated by cross-talk of CCK1R and ObR in the vagal afferent neurons. It was found silencing the STAT3 gene abolished synergistic actions on neuronal firing and co-treatment with CCK-8 and leptin increased p-STAT3. Further silencing Src and PI3K genes prevented p-STAT3 increase. As such it was suggested vagal CCK1R and ObR synergistic action leads to increased p-STAT3 causing potassium channel closure and neuronal firing, due to the interaction of CCK/Src/PI3K and leptin/JAK2/PI3K/STAT3 signalling

pathways (Heldsinger *et al.*, 2011). Therefore, CCK1R agonism, through A71623, and leptin could demonstrate synergistic effects. Therefore, this chapter aims to identify if the synergistic action of CCK and leptin extends beyond satiety functions to neuroprotective actions. As such it will explore low-dose co-treatments of leptin or leptin$_{116\text{-}130}$ with CCK1R agonist A71623 initially on undifferentiated and, after establishing synergistic protection, differentiated SHSY-5Y cells to assess the effectiveness of a co-treatment.

AD is an age-related disease which causes increased pathology and cognitive decline over time. As such, in addition to their synergistic relationship, the efficacy of A71623, leptin and leptin$_{116\text{-}130}$ over time must be explored. A prominent factor which may impact the usefulness of these compounds as treatments is changes in receptor expression with age. Previous work has suggested ObRa does but ObRb does not change with age in humans from analysis of peripheral blood mononuclear cells (Roszkowska-Gancarz *et al.*, 2015), however, little work has been done on the expression of CCK receptors in old age in humans. As such, I used an ageing SHSY-5Y model to assess changes in receptor expression and compared these changes with the common laboratory model, the rat, and with a species which naturally acquires AD-like decline, the cat. Feline cognitive dysfunction syndrome (CDS) occurs with age in cats and may be considered an Alzheimer's-like neurodegenerative disease (Gunn-Moore, 2011). Within CDS Aβ deposits and tau aggregation have been identified in feline brains which occur naturally (Gunn-Moore *et al.*, 2006), unlike rats or mice where such pathologies must be artificially modelled. By contrasting the human cell line to these two animal models I can consider the efficacy of their use and by contrasting patterns suggest how age and disease states may change these receptors.

Finally, I tested the ability of A71623, leptin and leptin$_{116\text{-}130}$ to elicit a survival response in neurons at different points on the ageing pathway by trialling a long-term low-dose treatment of Aβ. I employed cell viability assays to assess if exposure of fully differentiated SHSY-5Y neurons to Aβ over three weeks causes a gradual increase in cell death. Further I simultaneously treated these cultures

with A71623, leptin or leptin$_{116-130}$ in the presence or absence of Aβ to investigate toxicity or neuroprotective abilities over time.

7.2 Co-administration of leptin and A71623 decreases LDH release following SGD in undifferentiated SHSY-5Y cells.

Preliminary experiments indicated the protective abilities of treatments were undetectable at: 0.4nM A71623, 0.01nM leptin and 0.01nM leptin$_{116-130}$. As such these treatment concentrations were explored for additive protective effects using CV and LDH assays and demonstrated visually with representative photomicrographs (**Figure 50**).

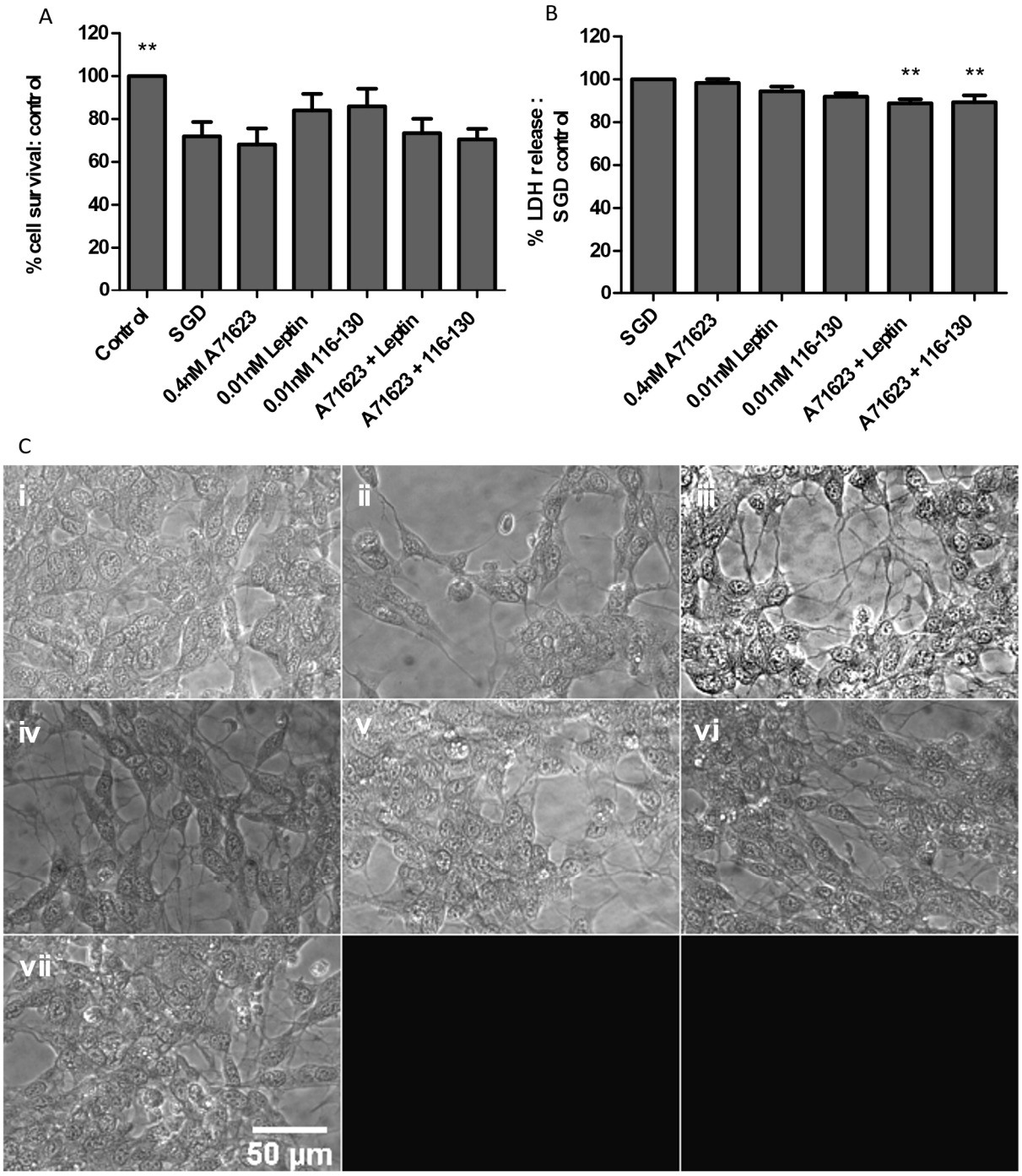

Figure 50. Low dose co-administration of leptin or leptin$_{116-130}$ and A71623 on undifferentiated SHSY-5Y cells under SGD. Graphs show mean +SEM. Significance on graphs relative to SGD condition are denoted by asterisks. A – shows mean percentage cell survival relative to untreated control as detected by CV assay. Only control shows higher cell survival compared to SGD condition ($H_{(6)}$=20.48, p=0.0023, n=7). B – shows mean percentage LDH release relative to SGD control as detected by LDH assay. Only combined treatments of low dose A71623 with leptin or leptin$_{116-130}$ show reduced LDH release ($F_{(5,34)}$=5.297, p=0.0011, n=7). C – Photomicrographs show representative images of control (i), SGD (ii), 0.4nM A71623 (iii), 0.01nM leptin (iv), 0.01nM leptin$_{116-130}$ (v), A71623 + leptin (vi) and A71623 + leptin$_{116-130}$ (vii) treated cells.

The CV assay (n=7) showed only the untreated control condition had higher percentage cell survival compared to SGD condition (28.08 ± 0 % $p<0.01$). However, the LDH assay (n=7) showed both co-treatments (A71623 with leptin 11.17 ± 5.163%, $p<0.01$; and A71623 with $leptin_{116-130}$ 10.66 ± 8.537%, $p<0.01$) had significantly less LDH release than SGD alone. In the photomicrographs the SGD condition alone has less viable cells in relation to control. Each treatment (0.4nM A71623, 0.01nM leptin and 0.01nM $leptin_{116-130}$) have less viable cells than the control condition and slightly more than SGD treatment alone. Co-treatments (A71623 + leptin and A71623 + $leptin_{116-130}$) appear to have more viable cells than SGD but differences from individual treatments are harder to judge.

During mitosis cell attachment to the culture plates substrate is reduced as they become rounded during cell division (Marchesi *et al.*, 2014). As the CV assay is reliant on healthy cells remaining attached to the substrate it is possible with significantly increased mitosis these cells would be washed away during the assay and CV assay readings would decrease. Therefore, cells were plated on coverslips and stained with DAPI in order to assess numbers of mitotic and apoptotic cells in each condition (**Figure 51**).

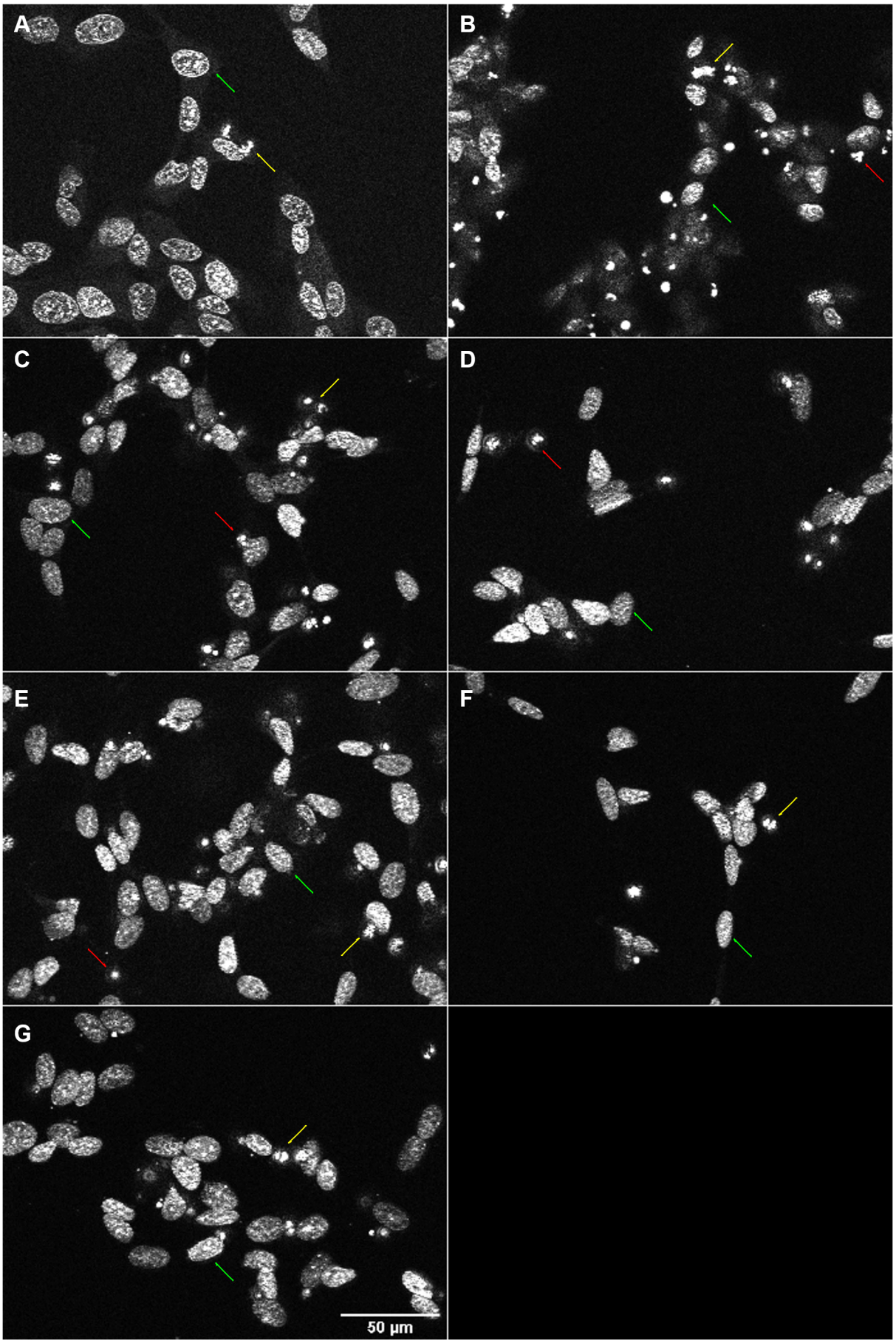
A
B
C
D
E
F
G
50 µm

Figure 51. DAPI stained nuclei from undifferentiated SHSY-5Y cells treated with A71623, leptin, leptin$_{116-130}$ or in combination under SGD conditions. Images show cells under control (A), SGD (B), 0.4nM A71623 (C), 0.01nM leptin (D), 0.01nM leptin$_{116-130}$ (E), A71623 + leptin (F), and A71623 + leptin116-130(G) conditions. Where possible green arrows indicate an example of a normal cell, yellow arrows a mitosing cell and red arrows an apoptotic cell in each condition.

In order to gain a quantitative measure of apoptotic and mitotic cell numbers in each condition, the number of normal, mitotic and apoptotic cells in each image were counted and calculated as a percentage of the total cell number (**Figure 52**).

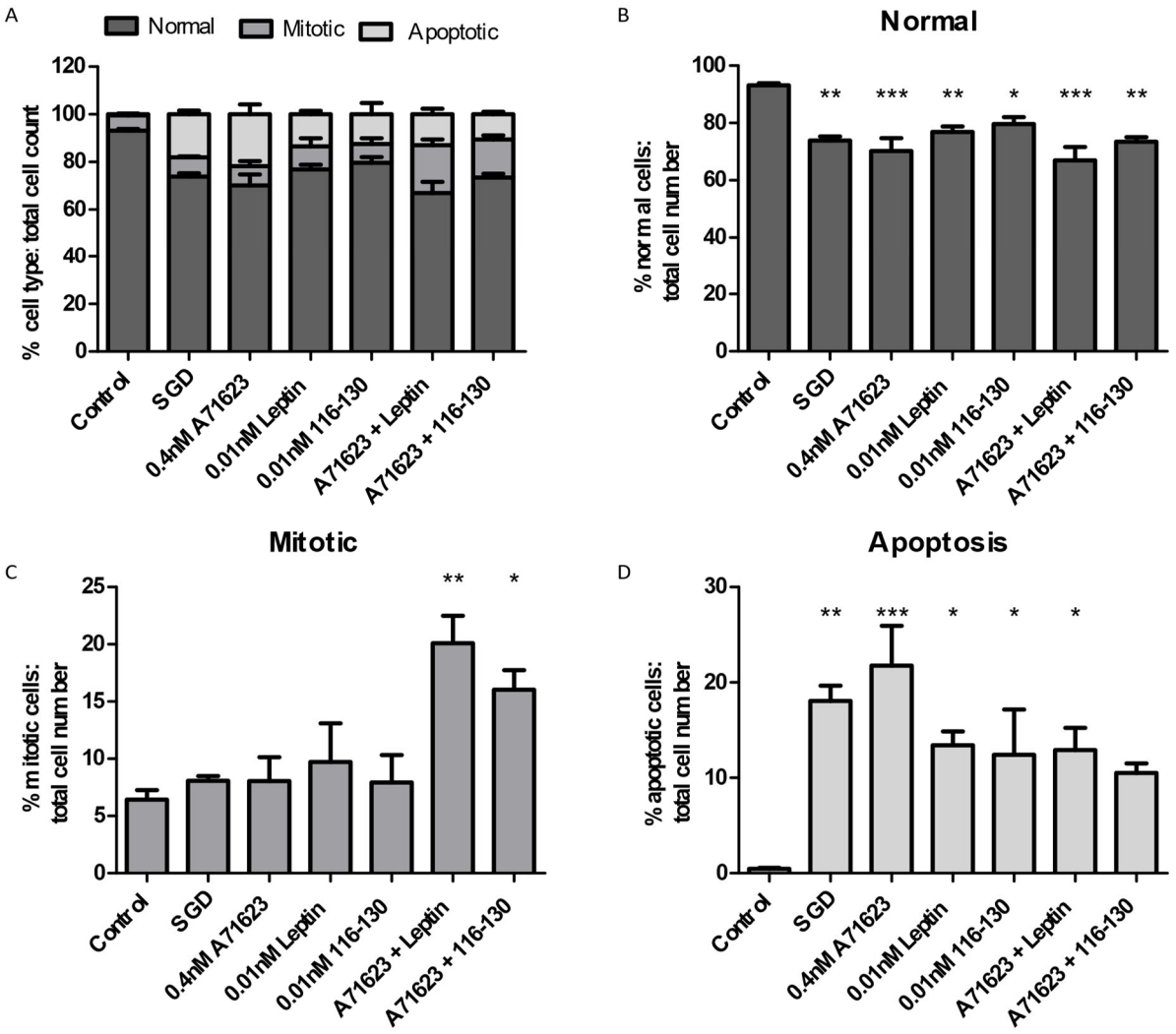

Figure 52. Combined A71623 and leptin or leptin$_{116-130}$ treatment increases mitosis and apoptosis under SGD conditions in undifferentiated SHSY5Y cells. Graphs show mean +SEM. Significance relative to control is denoted by asterisks. A – Stacked bar graph shows the pattern of percent normal, mitotic and apoptotic cells across treatment conditions. B – Average percent normal cell counts relative to total cell number per image shows all conditions had lower numbers of normal cells compared to control ($F_{(6,14)}$=8.876, p=0.0004, n=3). C – Shows the mean percentage mitotic cell counts are higher in co-treatment conditions relative to control ($F_{(6,14)}$=5.954, p=0.0029, n=3). D – demonstrates that all

treatment conditions except A71623 + leptin$_{116-130}$ had higher percentage apoptotic cells compared to the control condition ($F_{(6,14)}$=6.084, p=0.0026, n=3).

The representative images and the stacked bar graph (n=3) indicate decreased total number of normal cells and increased apoptotic cells across all conditions relative to control. Further it suggests an increase in mitotic cells in the co-treatments. Data was separated into normal, mitotic and apoptotic for statistical analysis. Data from cells termed normal, showed all conditions had decreased percent normal cell count compared to the control condition (SGD 19.26 ± 1.394%, p<0.01; 0.4nM A71623 22.92 ± 4.536%, p<0.001; 0.01nM leptin 16.25 ± 1.945%, p<0.01; 0.01nM leptin$_{116-130}$ 13.46 ± 2.402%, p<0.05; A71623 + leptin 26.12 ± 4.647%, p<0.001; A71623 + leptin$_{116-130}$ 19.65± 1.607%, p<0.01). This indicates all conditions had fewer cells that were not undergoing apoptosis or mitosis in comparison to the control condition. The mitotic cell data demonstrated co-treatments had significantly increased mitotic cells in comparison to control (A71623 + leptin 13.67 ± 2.380%, p<0.01; A71623 + leptin$_{116-130}$ 9.59± 1.705%, p<0.05) and apoptotic cell counts in all conditions except the co-treatment of A71623 and leptin$_{116-130}$ had significantly increased apoptosis relative to untreated control (SGD 17.62 ± 1.595%, p<0.01; 0.4nM A71623 21.30 ± 4.175%, p<0.001; 0.01nM leptin 12.96 ± 1.441%, p<0.05; 0.01nM leptin$_{116-130}$ 11.96 ± 4.742%, p<0.05; A71623 + leptin 12.46 ± 2.336%, p<0.05).

Altogether this shows SGD induced apoptosis in undifferentiated SHSY-5Y cells and neither low dose treatments of A71623, leptin or leptin$_{116-130}$ nor co-treatments of A71623 with leptin could prevent this, but A71623 with leptin$_{116-130}$ did. Further both co-treatments showed a significant increase in mitosis. This suggests co-treatment of A71623 and leptin or leptin$_{116-130}$ can induce mitosis.

7.3 Co-administration of A71623 with leptin$_{116-130}$ but not leptin protects against copper-induced toxicity in differentiated SHSY-5Y cells.

As co-treatment of A71623 with leptin or leptin$_{116-130}$ induced mitosis in undifferentiated SHSY-5Y cells and A71623 with leptin$_{116-130}$ did not show significantly increased apoptosis. The protective effects of co-treatment post-differentiation in the presence of a mitotic inhibitor were investigated. Preliminary experiments suggested the protective abilities of treatments individually were

undetectable against copper toxicity in differentiated SHSY-5Y cells at: 0.08nM A71623, 0.01nM leptin and 0.01nM leptin$_{116-130}$. As such viability was established using CV and LDH assays using these concentrations (**Figure 53**).

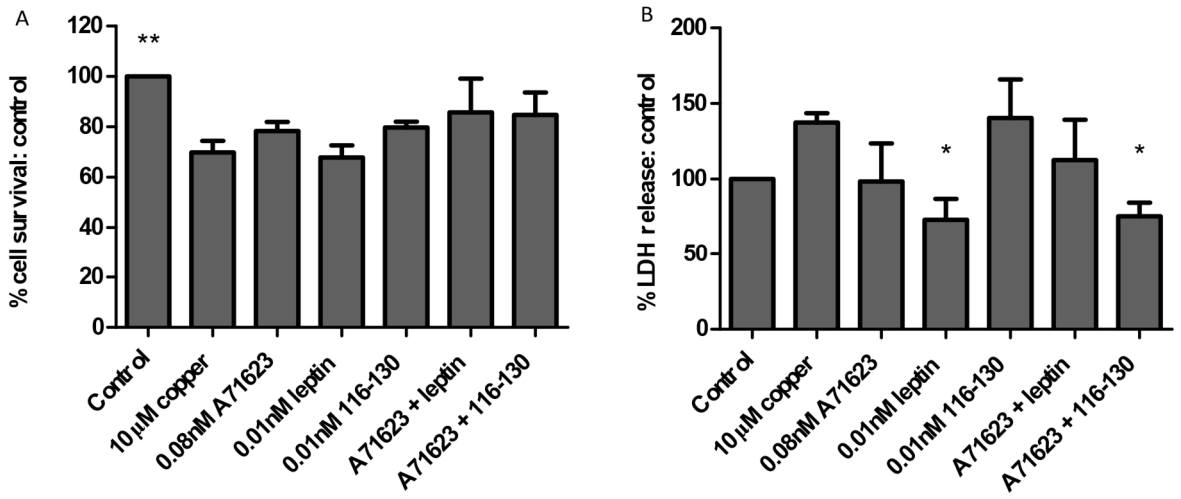

Figure 53. Low dose co-treatments of leptin or leptin$_{116-130}$ and A71623 on differentiated SHSY-5Y cells under copper toxicity. Graphs show mean +SEM. Significance on graphs relative to copper condition are denoted by asterisks. A – Shows mean percentage cell survival relative to untreated control as detected by CV assay. Only control shows higher cell survival compared to copper condition ($H_{(6)}$=20.60, p=0.0022, n=8). B – Shows mean percentage LDH release relative to copper control as detected by LDH assay. Only treatments of low dose leptin and co-treatment of A71623 with leptin$_{116-130}$ show reduced LDH release ($H_{(6)}$=15.44, p=0.0171, n=8).

The CV data (n=8) demonstrated that control had significantly increased cell survival compared to the copper treated condition (30.16 ± 0%, p<0.001) but no other differences were found. For the LDH data (n=8) 0.01nM leptin (64.22 ± 13.82%, p<0.05) and A71623+leptin$_{116-130}$ (61.81 ± 8.875%, p<0.05) had significantly decreased LDH release compared to the copper control. As such these results may indicate the combined treatment of A71623 with leptin$_{116-130}$ but not with leptin can produce additive protective effects. A firm conclusion is hard to draw from this data however, as no significant protective effect in the A71623 + leptin$_{116-130}$ data from the CV assay was seen.

7.4 Co-administration of A71623 with leptin or leptin$_{116-130}$ does not increase phosphorylation of STAT3 or Akt.

As combined treatments of A71623 with leptin$_{116-130}$ prevents membrane rupture in differentiated SHSY-5Y neurons the signalling pathways of co-administration were compared to

individual administration. This allowed investigation of an additive effects and differences in the signalling patterns in the two co-treatments. Leptin and leptin$_{116-130}$ signal through the STAT3 and Akt pathways (see 5.4) and A71623 signals through STAT3 (see 6.9). Previous work has suggested both of these pathways are involved in the synergistic action of CCK (through CCK1R) and leptin (Heldsinger *et al.*, 2011). Therefore, ELISA was used to assess if there was an increase in signalling through these pathways, as demonstrated by increased phosphorylation of STAT3 and Akt, with combined treatments of A71623 with leptin or leptin$_{116-130}$ (Figure 54). Assays were carried out after 96h in treatment to mirror treatment lengths used in the viability assays.

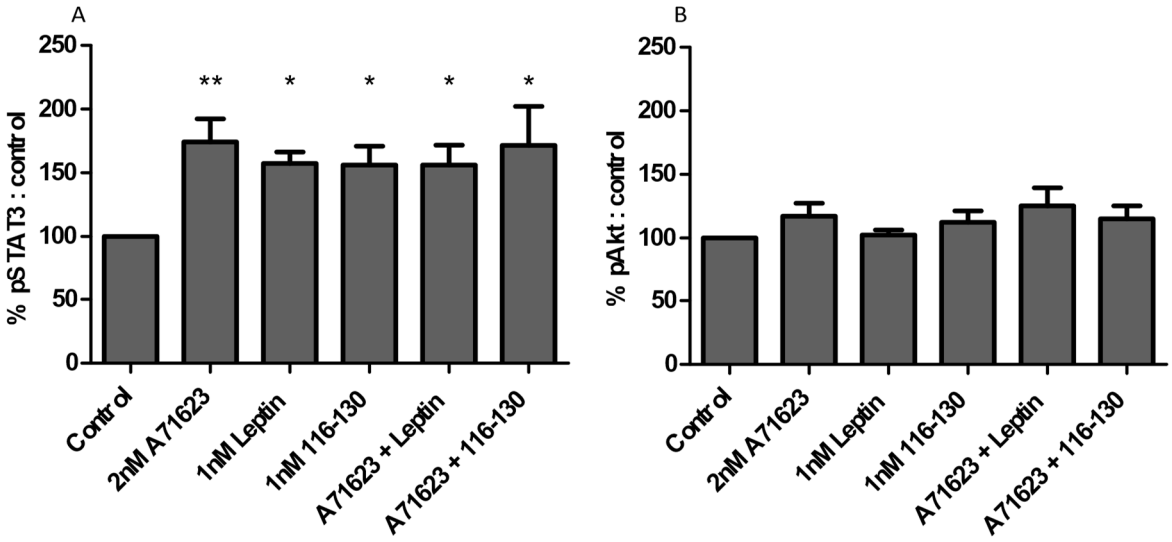

Figure 54. Signalling of co-treatments of A71623 with leptin or leptin$_{116-130}$. Graphs show mean + SEM. A - Shows percent pSTAT3 in conditions treated with A71623, leptin, leptin$_{116-130}$ or co-treatments relative to an untreated control. All treatments show significant increases in pSTAT3 ($H_{(5)}$=17.91, p=0.0031, n=7). B – Shows percent pAkt in conditions treated with A71623, leptin or leptin$_{116-130}$ or co-treatments relative to an untreated control. No significant changes in pAkt levels were found ($H_{(5)}$=4.27, p=0.5113, n=8).

All conditions had a significant increase in pSTAT3 compared to control (2nM A71623: 74.2 ± 18.22%, p<0.01; 1nM leptin: 57.3 ± 9.060%, p<0.05; 1nM leptin$_{116-130}$: 56.2 ± 14.80%, p<0.05; A71623 + leptin: 56.1 ± 15.65%, p<0.05; A71623 + leptin$_{116-130}$ 71.6 ± 30.70%, p<0.05; n=8). However, there was no significant increase from A71623, leptin or leptin$_{116-130}$ treatments alone compared to co-treatments. This shows that A71623, leptin and leptin$_{116-130}$ all signalled through the STAT3 pathway but there was no additive effect from combined treatment to increase signalling further.

Data from the pAkt ELISAs show there were no significant differences between treatments. This shows that at the time point examined A71623, leptin nor leptin$_{116-130}$ were signalling through the Akt pathway and there was no additive effect of combined treatment. Altogether this indicates that whilst protective abilities of A71623, leptin and leptin$_{116-130}$ are mediated through STAT3 their co-administration does not further employ this pathway and long-term Akt is not involved in signalling for any treatment.

7.5 The effects of co-administration of A71623 with leptin or leptin$_{116-130}$ on AD biomarkers.

Previous work shows Leptin modulates biomarkers of AD (Doherty *et al.*, 2013; Niedowicz *et al.*, 2013; Platt *et al.*, 2016), CCK1R agonism through A71623 can modulate p-tau levels under AD relevant conditions (see 6.10) and co-treatments of A71623 and leptin or leptin$_{116-130}$ produce protective effects (see 7.2 and 7.3). As such co-treatments of A71623 with leptin or leptin$_{116-130}$ were investigated for modulation of AD biomarkers. Levels of p-tau, tau, ABAD, APP, endophilin 1 and presenilin 1 (as previously described, see 5.5) were detected by ELISA in differentiated SHSY-5Y cultures treated for 96h. For these experiments 3 out of 8 experimental repeats were used, following the previously described inclusion criteria (see 5.5).

7.5.1 Changes to expression of AD biomarkers by co-treatment of A71623 and leptin.

Cultures were treated with a combination of 1µM Aβ, 2nM A71623 and 1nM leptin and protein extracted for ELISA to assess changes in AD-linked biomarkers (**Figure 55**). These concentrations of A71623 and leptin have previously shown neuroprotective effects (see 5.3 and 6.8) but separately no significant effects on chosen biomarkers (see 5.5 and 6.10). Due to the synergistic nature of leptin and CCK co-administration of these concentrations may demonstrate modulative effects unseen with separate treatment.

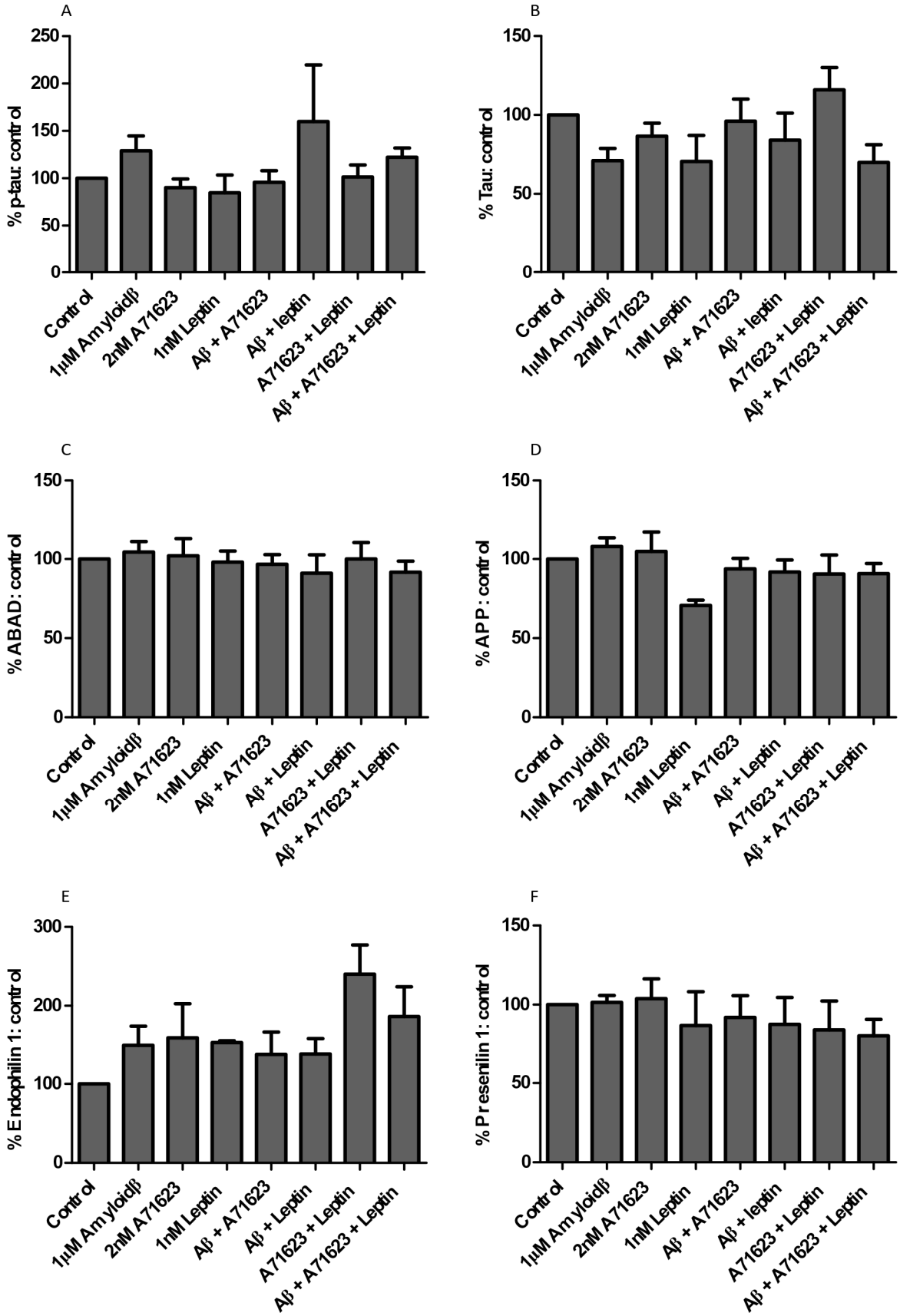

Figure 55. ELISAs for AD biomarkers in cells treated with Aβ, A71623 and leptin. Graphs show mean percentage antibody relative to control + SEM. A – Shows that Aβ increased p-tau and combined

treatment was not able to ameliorate this effect ($H_{(7)}$=8.682, p=0.2763, n=3). B – Aβ may have slightly decreased tau levels, similarly, levels are decreased in A71623 with leptin treatment in the presence of Aβ ($F_{(7,16)}$=1.804, p=0.1555, n=3). C - No pronounced changes in ABAD can be seen, although co-treatment may demonstrate slightly reduced levels ($F_{(7,16)}$=0.3314, p=0.9281, n=3). D – Shows leptin and co-treatments demonstrate slightly reduced APP levels ($F_{(7,16)}$=2.151, p=0.0969, n=3). E – All treatments show a suggested increase in endophilin 1 relative to control, especially co-treatments ($H_{(7)}$=10.54, p=0.1601, n=3). F – Shows there may be a trend towards decreased presenilin 1 level in the cultures treated with Aβ and A71623 with leptin ($F_{(7,16)}$=0.3959, p=0.8911, n=3).

No significant differences were found using for any biomarker examined (p-tau p=0.2763; tau p=0.1555; ABAD p=0.9281; APP p=0.0969; endophilin 1 p=0.1601; presenilin 1 p=0.8911; n=3 for all). These results indicate that no treatment with A71623, leptin or a co-treatment was able to modulate AD biomarkers in these cultures, further Aβ treatment alone also could not modulate the chosen biomarkers.

7.5.2 Changes in expression of AD biomarkers in co-treatments of A71623 and leptin$_{116\text{-}130}$

Cultures were treated with a combination of 1μM Aβ, 2nM A71623 and 1nM leptin$_{116\text{-}130}$ and protein extracted for ELISA to examine modulation of AD-linked biomarkers (**Figure 56**).

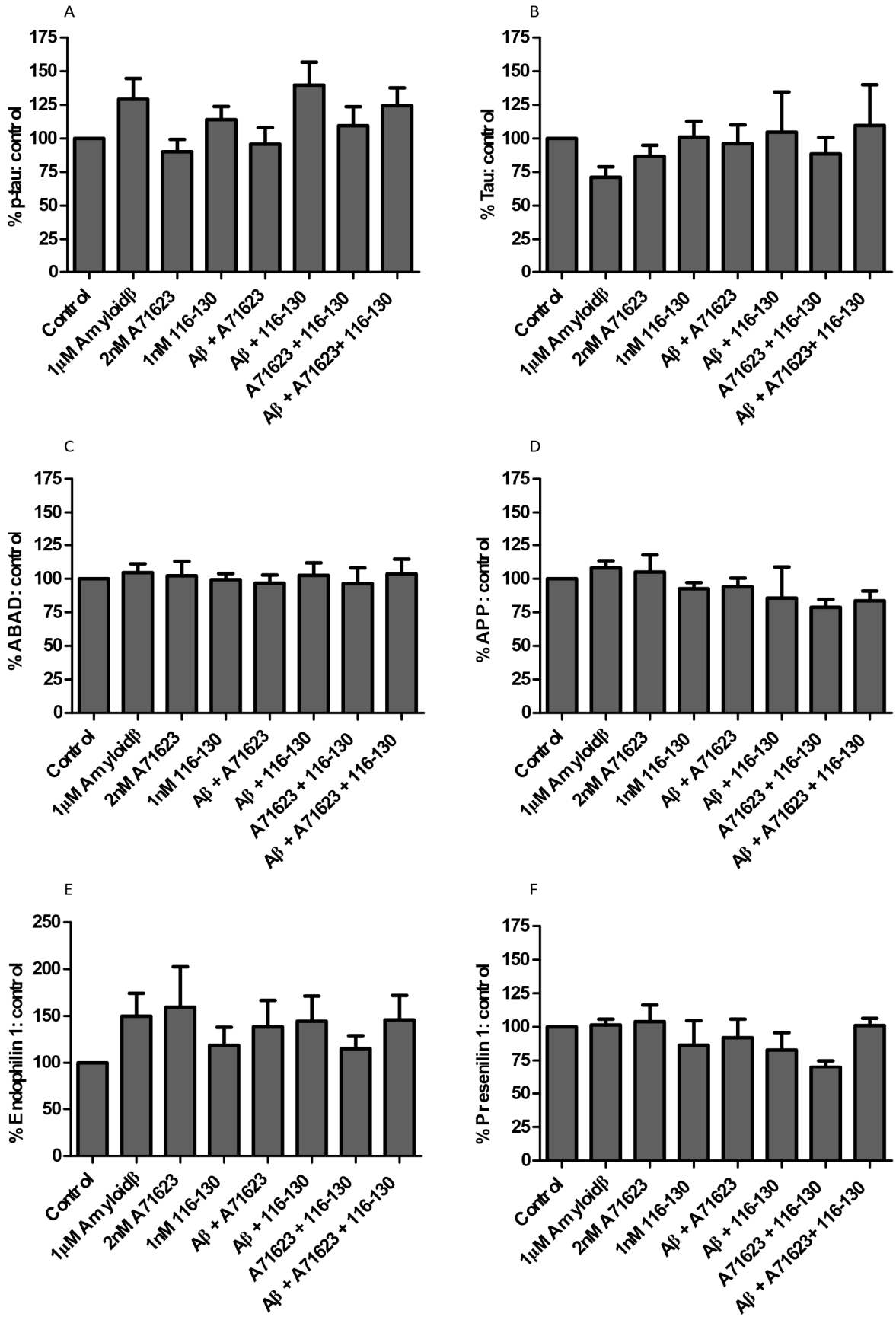

Figure 56. ELISAs for AD biomarkers in cells treated with Aβ, A71623 and leptin$_{116-130}$. Graphs show mean percentage antibody relative to control + SEM. A – Shows that Aβ increased p-tau and combined

treatment was not able to ameliorate this effect ($F_{(7,16)}$=1.942, p=0.1286, n=3). B – Aβ may have slightly decreased tau levels but A71623 with leptin$_{116-130}$ treatment in the presence of Aβ shows increased levels ($H_{(7)}$=5.329, p=0.6198, n=3). C - No pronounced changes in ABAD can be seen ($F_{(7,16)}$=0.1316, p=0.9943, n=3). D – Shows leptin$_{116-130}$ and co-treatments have slightly reduced APP levels ($F_{(7,16)}$=0.9902, p=0.4722, n=3). E – All treatments show a suggested increase in endophilin 1 relative to control ($H_{(7)}$=4.234, p=0.7525, n=3). F – Shows no changes presenilin 1 level in the cultures treated with Aβ and A71623 with leptin$_{116-130}$ compared to Aβ treatment alone ($F_{(7,16)}$=1.213, p=0.3512, n=3).

No significant differences were found for any biomarker investigated (p-tau p=0.1286; tau p=0.6198; ABAD p=0.9943; APP p=0.4722; endophilin 1 p=0.7525; presenilin 1 p=0.3512; n=3 for all). These results indicate that no treatment with A71623, leptin$_{116-130}$ or a co-treatment was able to modulate AD biomarkers in these cultures, further Aβ treatment alone also could not modulate the chosen biomarkers.

Co-administration of A71623 with leptin or leptin$_{116-130}$ induces mitosis in undifferentiated SHSY-5Y cells. Further, A71623 with leptin$_{116-130}$ did not significantly increase apoptosis under SGD in undifferentiated cells and decreased membrane rupture caused by copper toxicity in differentiated cells. This demonstrates synergistic abilities in co-administration with a neuroprotective effect more likely from A71623 with leptin$_{116-130}$. However, no difference in signalling pathways between single treatments and co-administration could be found in STAT3 and Akt phosphorylation indicating another pathway may be involved in co-treatment. Finally, no significant synergistic action on modulation of AD biomarkers was identified. However, Aβ treatment was unable to upregulate these biomarkers, as such these results are inconclusive. Though the mode of action remains to be elucidated, this demonstrates a potential benefit to co-treatment of A71623 and leptin$_{116-130}$.

7.6 CCK1R AND OBR EXPRESSION VARIES WITH BRAIN REGION, AGE AND DISEASE.

AD is a disease of ageing. Expression levels of receptors can change with age and therefore efficacy of a treatment requiring these receptors may also be affected. As such expression of ObR and CCK1R with age were explored using both cellular and animal models. I aimed to discover how receptor expression changes with age in the human SHSY-5Y model and compare this to the common animal

model, the rat. This allows comparison to tissue from the brain which may have been differentially affected by age than isolated cultures. Further tissue can be gathered from rats across lifespan knowing they have been unaffected by disease and due to the inbred nature laboratory models genetic idiosyncrasies which may impact results should be minimised. However, rodent models are not naturally affected by AD, as such while receptor changes may have similar patterns to humans, we cannot explore how AD may also impact these receptors in a naturalistic way. With age cats may acquire the AD-like feline cognitive dysfunction syndrome (CDS) which demonstrates Aβ deposits and tau aggregation similarly to AD in humans (Gunn-Moore *et al.*, 2006; Gunn-Moore, 2011). As such analysis of how receptor expression is affected across these models with age and an AD-like state was undertaken.

7.6.1 ObR expression shows no changes with age in SHSY-5Y neurons or rat cortex but changes with age and disease across regions in the cat brain.

Exploration of the leptin receptor ObR was undertaken to assess changes with age which may be vital to a leptin peptide treatment. Protein was extracted from cell cultures across six time points (undifferentiated, differentiated, 1, 2, 3 and 4 weeks post differentiation) and rat cortex at four ages (juvenile, postweaning, adult and aged) for analysis of ObR expression by ELISA (Figure 57). Rat brains were donated by Dr J. Harvey, University of Dundee.

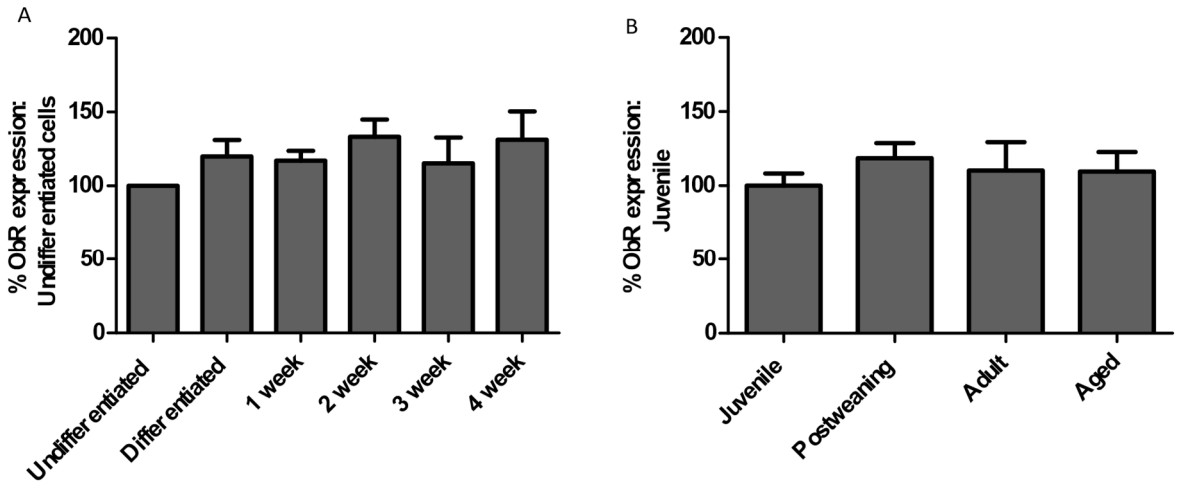

Figure 57. ObR expression across age in SHSY-5Y cells and rat cortex. A- Graph show mean + SEM percent ObR expression relative to undifferentiated SHSY5Y cells. No significant changes in receptor

expression occur with age ($F_{(5,36)}$=0.9002, p=0.4916, n=7). B - Graph show mean + SEM percent ObR expression relative to Juvenile brains as measured by ELISA. ObR expression shows no change with age in the rat cortex ($F_{(3,12)}$=0.3221, p=0.8093, n=4).

The ELISA data from SHSY-5Y cells show no change in ObR expression (p=0.9744; n=7) a pattern which is mirrored in the rat cortex (p=0.8093; n=4). To compare the effects of an AD-like disease on receptor expression changes in ObR with age and in CDS were analysed in the cat brain by immunohistochemistry (sections donated by Professor D. Gunn-Moore, University of Edinburgh). Paraffin embedded brain sections of young cats (under 10 years old, mean=6.4 ± 2.5, n=5), old cats (over 10 years old, mean=14 ± 2.1, n=5) and cats with confirmed diagnosis of CDS (mean age=15.0 ± 3.8, n=5) were used. Four brain regions were stained per cat, which were: cerebellum, parietal, occipital and rostral. At least 2 images were taken from each region for each cat. Due to the layered structure of the cerebellum, analysis of these images focussed on specific cell types. Therefore, images were analysed for CTCF of Purkinje cells, number of positive stained Purkinje cells and % area of granular layer covered by positive staining (**Figure 58**). Purkinje cells are large neurons localised between the molecular and granule cell layers and as such are easily identifiable.

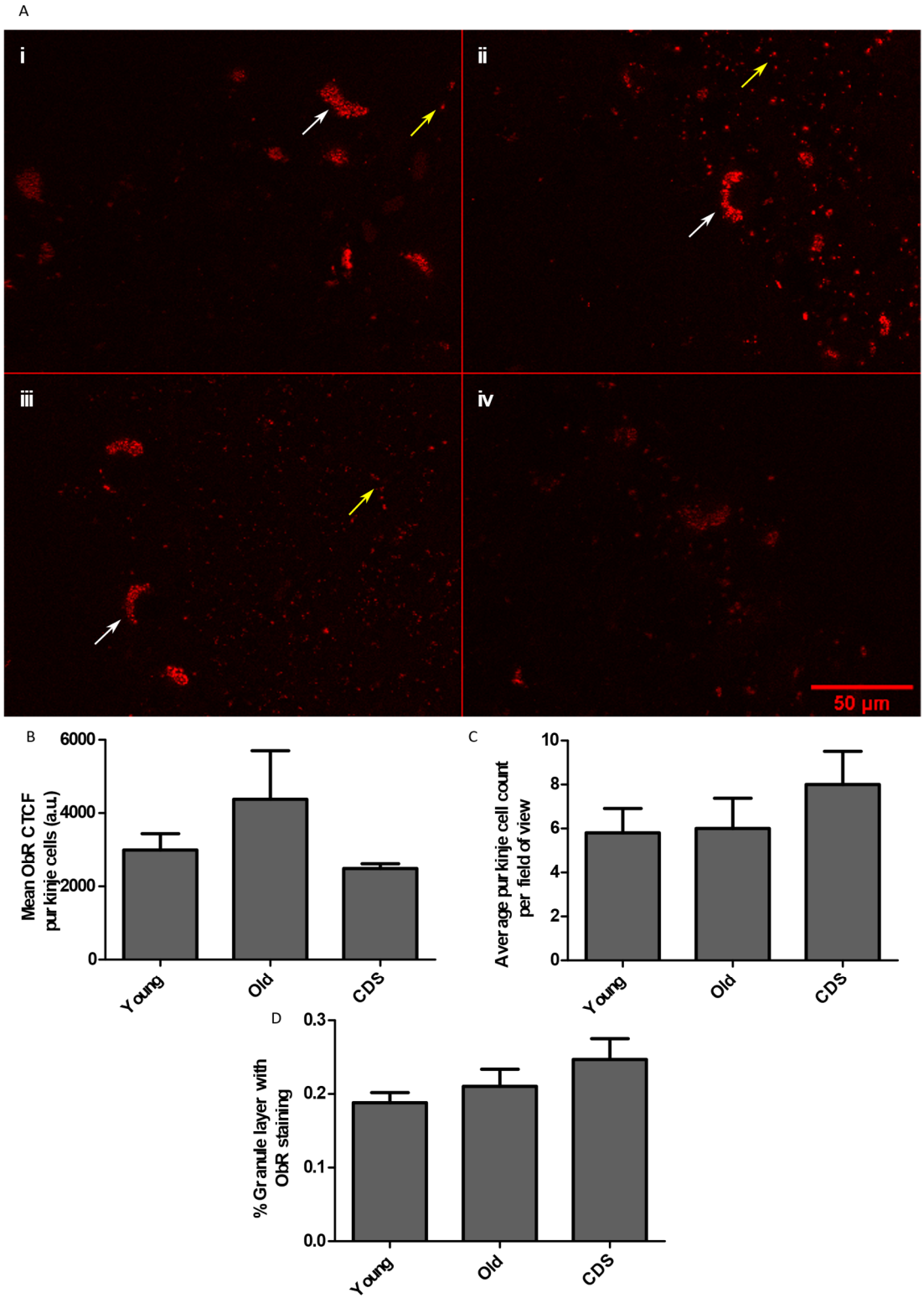

Figure 58. ObR staining in the cat cerebellum from young, old and CDS cats. Graphs show mean + SEM. A – Images show sections taken from the cerebellum of young (i), old (ii), and confirmed diagnosis CDS (iii) cats stained with (i, ii, iii) or without (iv) ObR primary antibody and all containing a Texas red secondary to detect receptor expression. B – Graph shows mean ObR CTCF as measured in positively stained Purkinje cells from the cerebellum for each group ($H_{(2)}$=0.8162, p=0.4652, n=5). C – Shows the average number of positively stained Purkinje cells per field of view for each group ($F_{(2,12)}$=0.1316, p=0.9943, n=5). D – Shows the average % area of positive staining in the granule layer

for each group ($F_{(2,12)}$=1.719, p=0.2205, n=5). White arrows indicate examples of Purkinje cells, yellow arrows show examples of granule cells.

The fluorescent images show all cats: young, old and CDS, demonstrated positive ObR staining in Purkinje and granule cells compared to the primary lacking control. Quantitative analysis shows no significant differences were found in CTCF of Purkinje cells (p=0.6771), average number of Purkinje cells per field of view (p=0.4652) or positive staining in the granular layer (p=0.2205). ObR expression did not significantly change with age or disease in the cat cerebellum.

The occipital sections were analysed by assessing CTCF, number of positive stained neurons per field of view and the % area of the image covered by positive staining (**Figure 59**).

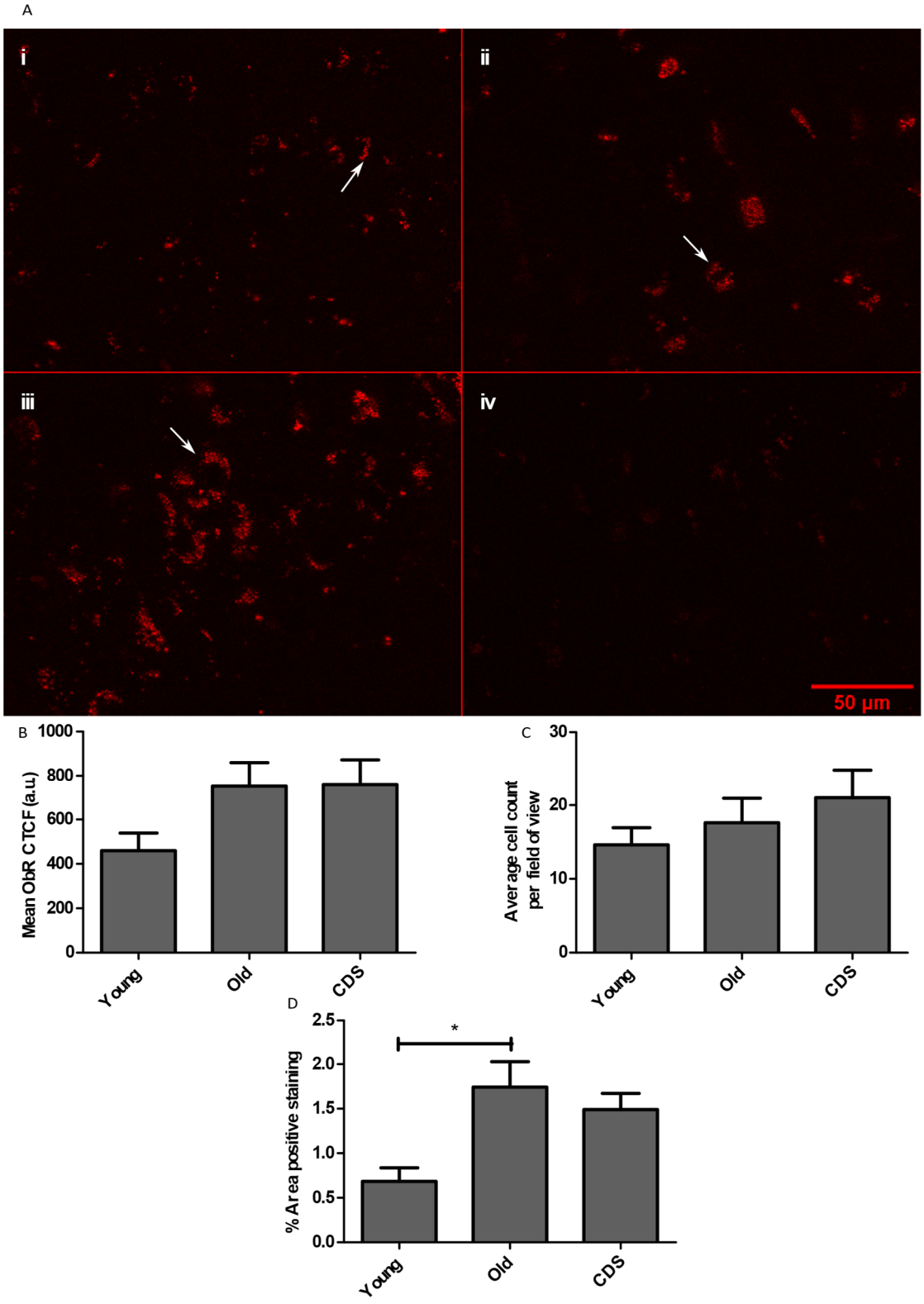

Figure 59. ObR staining in the occipital section of young, old and CDS cat brains. Graphs show mean + SEM. A – Images show sections taken from occipital sections of young (i), old (ii), and confirmed diagnosis CDS (iii) cat brains stained with (i, ii, iii) or without (iv) ObR primary antibody and all containing a Texas red secondary to detect receptor expression. B – Graph shows mean ObR CTCF as measured in positively stained neurons for each group ($F_{(2,12)}$=2.758, p=0.1034, n=5). C – Shows the average number of positively stained neurons per field of view for each group ($F_{(2,12)}$=0.9790,

p=0.4038, n=5). D – Shows the average % area of positive staining per field of view for each group ($F_{(2,12)}$=6.620, p=0.0115, n=5). White arrows show examples of positively stained neurons.

No significant differences in CTCF (p=0.1034) or positively stained cell counts (p=0.4038) were found. A significant change occurred in %area covered in positive staining (p=0.0115) with young cats (1.058 ± 0.1525%, p<0.05) demonstrating significantly less area of positive staining compared to old. No significant difference was found between CDS cats and either group. This suggests although ObR staining was not significantly brighter, nor were there significantly more cells with ObR staining, the area covered in ObR per field of view was greater in the old cats. This may imply cells are larger in older cats compared to young or there is greater extra-neuronal staining.

Analysis of the parietal sections also assessed CTCF, number of positive stained neurons per field of view and the % area of the image covered by positive staining (**Figure 60**).

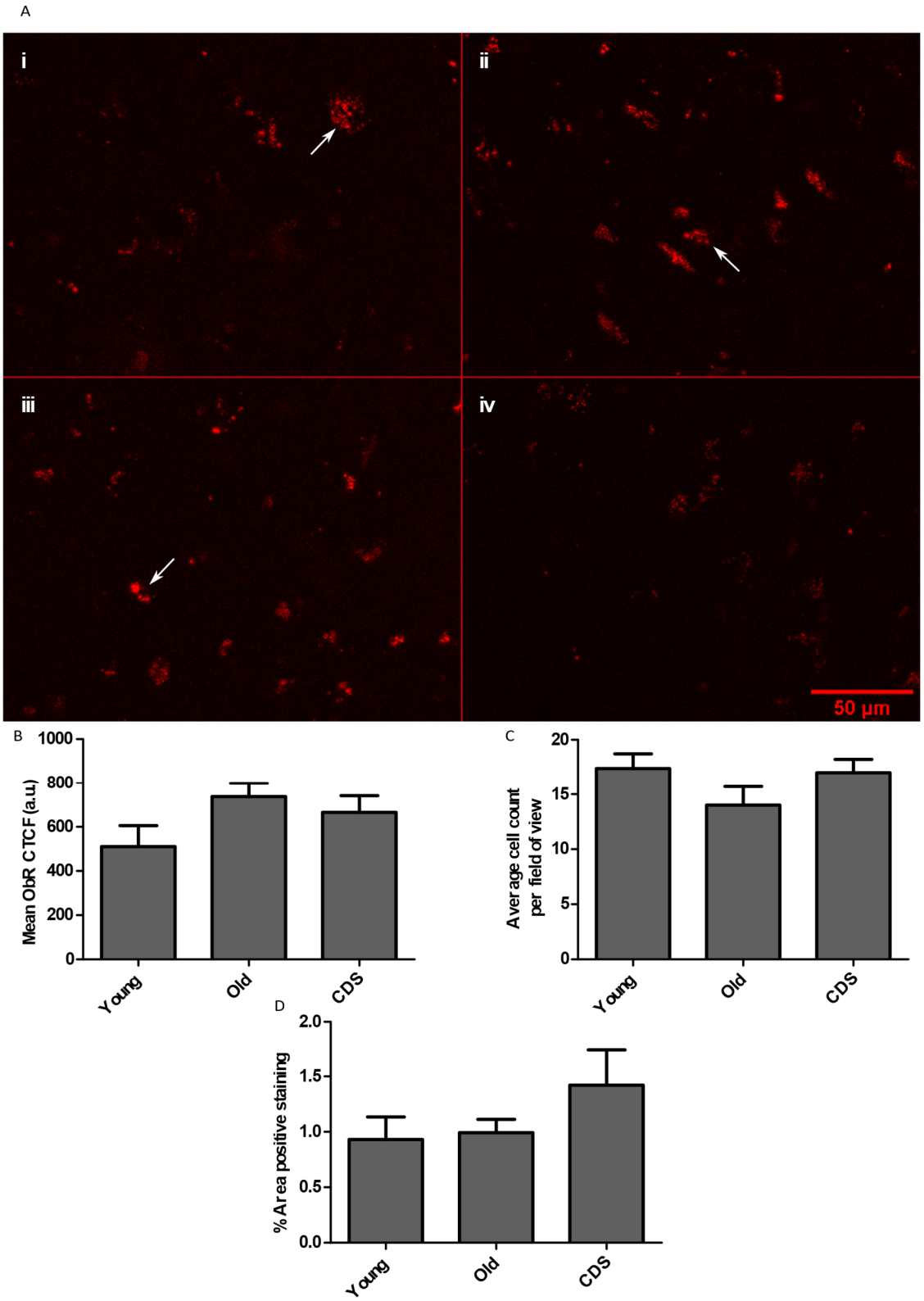

Figure 60. Parietal cat brain section ObR staining from young, old and CDS cats. Graphs show mean + SEM. A – Images show sections taken from parietal sections of young (i), old (ii), and confirmed diagnosis CDS (iii) cat brains stained with (i, ii, iii) or without (iv) ObR primary antibody and all containing a Texas red secondary to detect receptor expression. B – Graph shows mean ObR CTCF as measured in positively stained neurons for each group ($F_{(2,12)}$=2.150, p=0.1593, n=5). C – Shows the average number of positively stained neurons per field of view for each group ($F_{(2,11)}$=1.668, p=0.2329,

n=5). D – Shows the average % area of positive staining per field of view for each group ($F_{(2,12)}$=1.354, p=0.2950, n=5). White arrows show examples of positively stained neurons.

Quantitative analysis shows no significant differences in fluorescence between young, old or CDS cats (CTCF p=0.1593; positive cell counts p=0.2329; %area with positive staining p=0.2950). These results suggest no age or disease related changes in ObR in the parietal section of the cat brain.

Finally, rostral sections were analysed for CTCF, number of positive neurons and % area covered in positive staining (**Figure 61**).

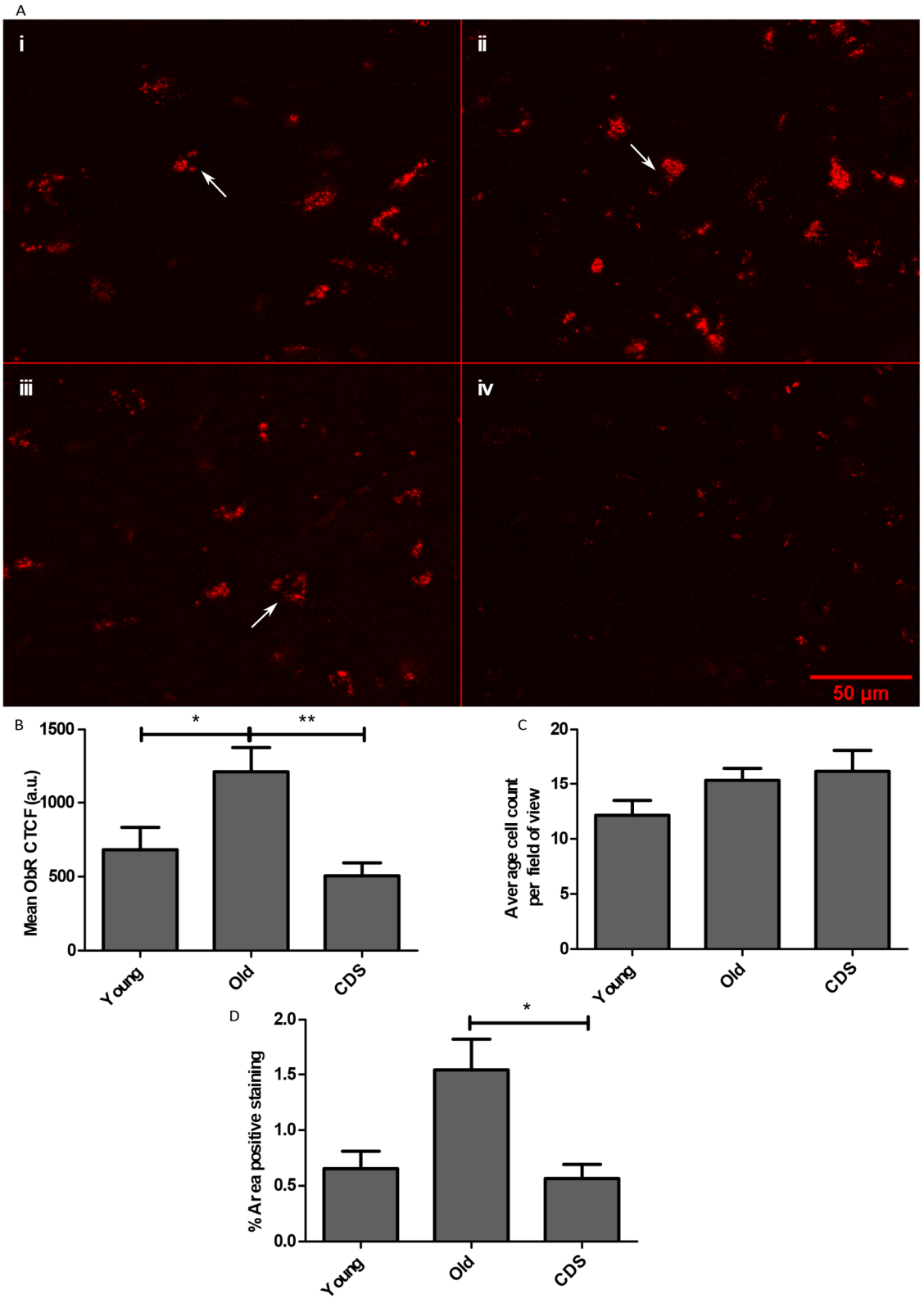

Figure 61. ObR staining in the rostral sections from young, old and CDS cat brains. Graphs show mean + SEM. A – Images show sections taken from rostral sections of young (i), old (ii), and confirmed diagnosis CDS (iii) cat brains stained with (i, ii, iii) or without (iv) ObR primary antibody and all containing a Texas red secondary to detect receptor expression. B – Graph shows mean ObR CTCF as measured in positively stained neurons for each group ($F_{(2,12)}$=7.147, p=0.009, n=5). C – Shows the average number of positively stained neurons per field of view for each group ($F_{(2,12)}$=2.091, p=0.1663,

n=5). D – Shows the average % area of positive staining per field of view for each group ($H_{(2)}$=8.074, p=0.0176, n=5). White arrows show examples of positively stained neurons.

CTCF analysis of the primary-containing images shows a significantly less fluorescence in young and CDS cats than old cats (young: 533.0 ± 151.7 a.u., p<0.05; CDS: 709.2 ± 87.31 a.u., p<0.01). Whilst there were no significant differences in the number of positively stained neurons across groups (p=0.1663) the %area with ObR positive staining shows CDS cats had significantly reduced area compared to old cats (0.9745 ± 0.1260%). No significant difference was detected between old and young cats. This data suggests that although the number of cells containing positive staining was not different between groups in old cats ObR staining was more intense within each positively stained cell relative to both young and CDS cats and that the area of the image covered in positive staining was greater in old cats relative to CDS cats. This means that there was greater ObR expression within cells in old cats and may suggest larger cells or greater extra-neuronal staining in old cats compared to CDS cats.

7.6.2 CCK1R expression decreases in SHSY-5Y and rat cortex with age but varies across age, disease and section in the cat brain.

Protein extracts from cell cultures and rat cortex were used for ELISA to assess changes in CCK1R expression with age (Figure 62).

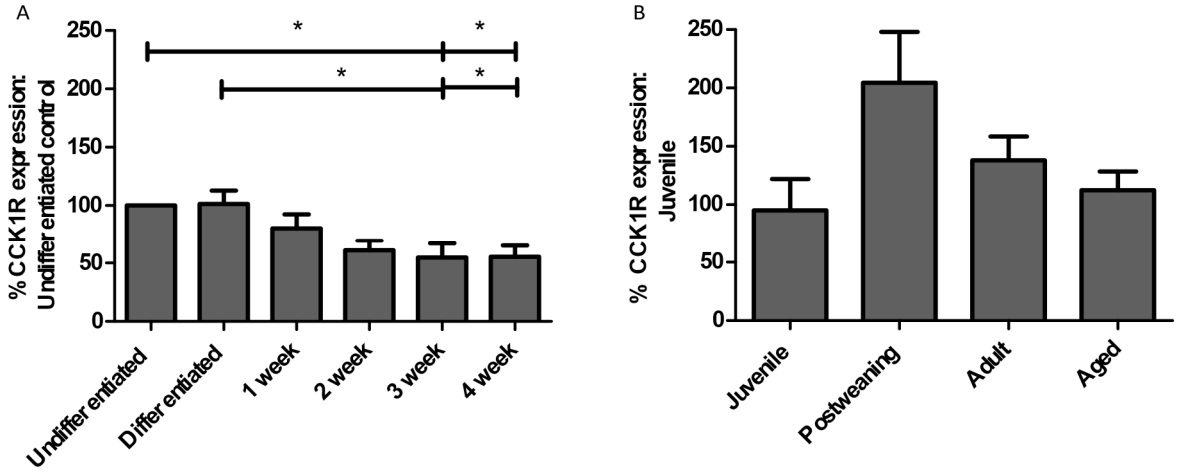

Figure 62. CCK1R expression across age in SHSY-5Y cells and rat cortex. A - Graph shows mean + SEM percent CCK1R expression relative to undifferentiated cells. Cells at 3- and 4-weeks post-differentiation show significantly reduced CCK1R expression relative to undifferentiated and differentiated cells ($F_{(5,32)}$=4.834, p=0.0021, n=7). B - Graph shows mean + SEM percent CCK1R

expression relative to Juvenile brains as measured by ELISA. CCK1R expression seems to indicate a potential peak in expression during the post-weaning age range with a decline in expression thereafter ($H_{(3)}$=5.559, p=0.1352, n=4).

SHSY-5Y cells show a significant change in CCK1R expression over time (p=0.0021; n=7). Cells at 3 weeks and 4 weeks post-differentiation had significantly less CCK1R expression compared to undifferentiated (3 weeks: 45.24 ± 12.15%, p<0.05; 4 weeks: 44.62 ± 9.782%, p<0.05) and differentiated cells (3 weeks: 46.68 ± 12.15%, p<0.05; 4 weeks: 46.06 ± 9.782%, p<0.05). Therefore, expression of CCK1R decreased with age. In the rat cortex no significant change in CCK1R expression was detected (p=0.1352; n=4 for each age).

Cat brains were explored using fluorescent immunohistochemical techniques to examine changes in CCK1R with age and CDS. A minimum of 3 images were taken for each region for each cat. Images from the cerebellum were analysed first for CTCF of Purkinje cells, number of positive stained Purkinje cells and % area of granular layer covered by positive staining (**Figure 63**).

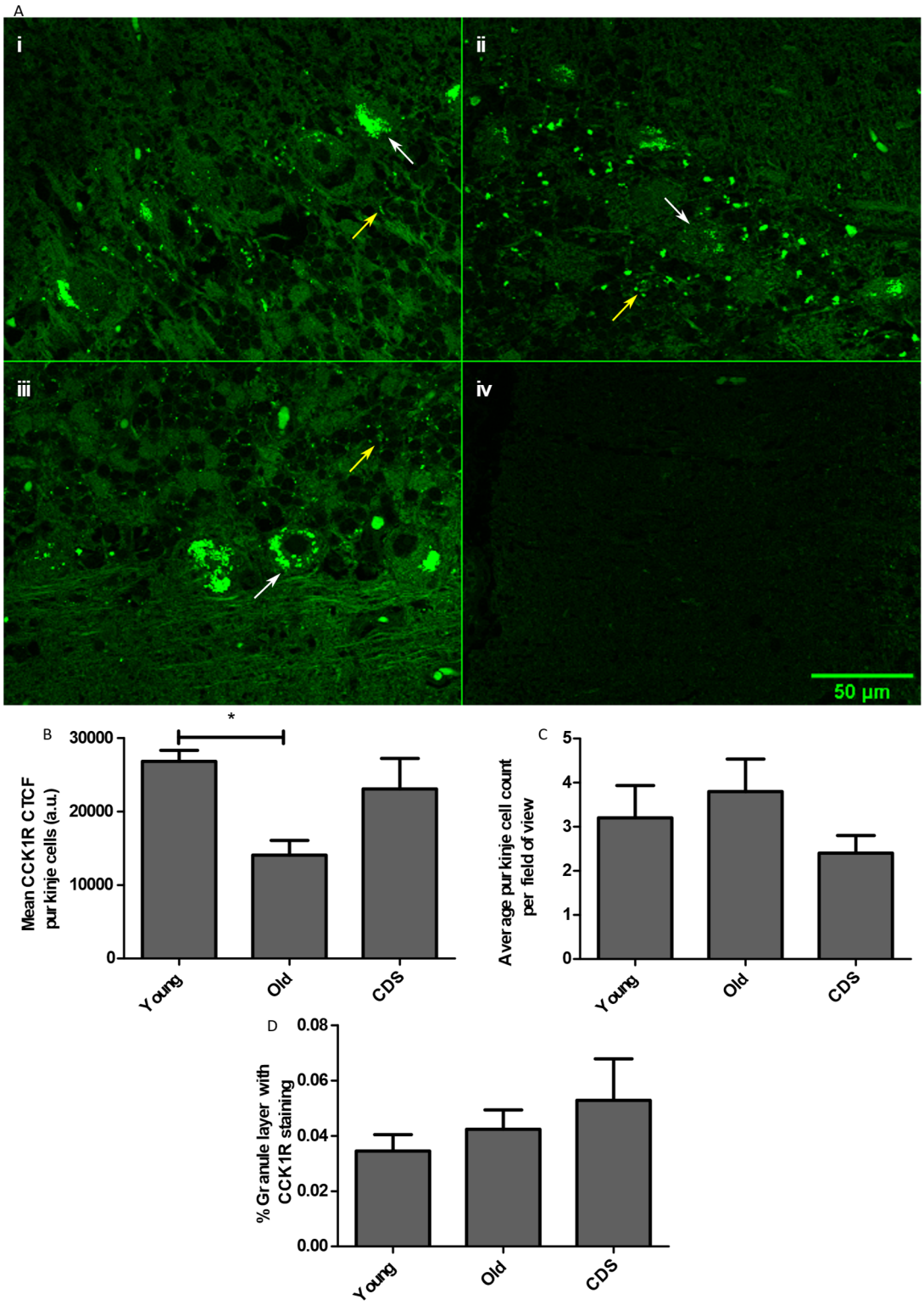

Figure 63. CCK1R staining for cerebellar Purkinje and granular cells in young, old and CDS cat brains. Graphs show mean + SEM. A – Images show sections taken from the cerebellum of young (i), old (ii), and confirmed diagnosis CDS (iii) cats stained with (i, ii, iii) or without (iv) CCK1R primary antibody and all containing a Dylight 488 secondary to detect receptor expression. B – Graph shows mean CCK1R CTCF as measured in positively stained Purkinje cells from the cerebellum for each group ($F_{(2,12)}$=4.827, p=0.0313, n=5). C – Shows the average number of positively stained Purkinje cells per field of view for each group ($F_{(2,11)}$=1.194, p=0.3367, n=5). D – Shows the average % area of positive staining in the

granule layer for each group ($F_{(2,12)}$=0.8157, p=0.4654, n=5). White arrows indicate examples of Purkinje cells, yellow arrows show examples of granule cells.

CTCF as measured from Purkinje cells in the cerebellum shows young cats had significantly higher CTCF than old cats (12730 ± 1501 a.u., $p<0.05$) but no significant difference was found between old and CDS cats. There were no significant changes in cell counts (p=0.3367) or % staining of granule layer (p=0.4654). This data suggests whilst all groups had similar numbers of Purkinje cells with CCK1R staining the intensity of staining within cells in the young cats was significantly greater than in the old cats, indicating great CCK1R expression.

Images from occipital sections were analysed for CTCF, number of positive stained neurons per field of view and the % area covered by CCK1R positive staining (**Figure 64**).

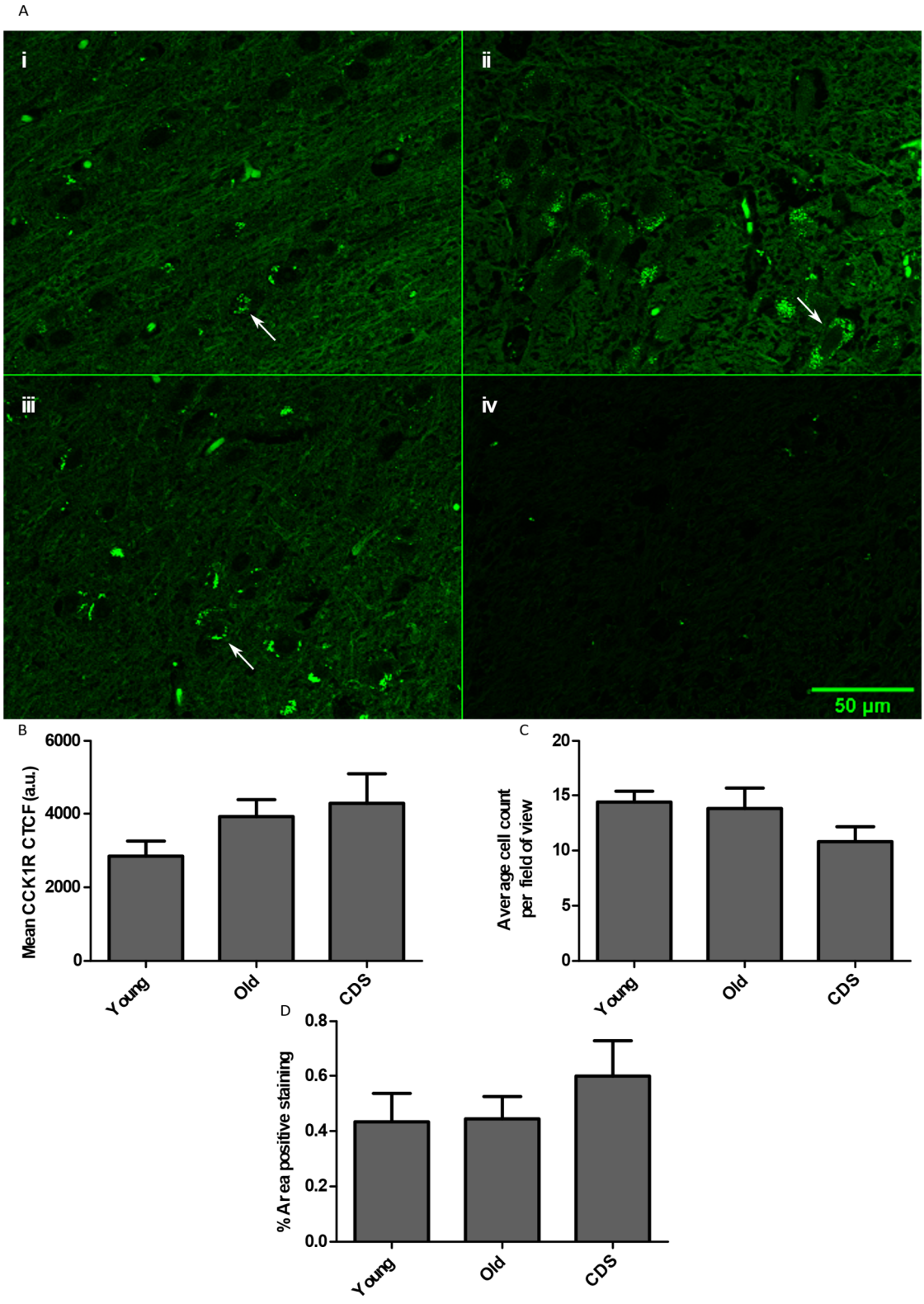

Figure 64. CCK1R staining in the occipital section of young, old and CDS cat brains. Graphs show mean + SEM. A – Images show sections taken from parietal sections of young (i), old (ii), and confirmed diagnosis CDS (iii) cat brains stained with (i, ii, iii) or without (iv) CCK1R primary antibody and all containing a Dylight 488 secondary to detect receptor expression. B – Graph shows mean CCK1R CTCF as measured in positively stained neurons for each group ($F_{(2,12)}$=1.572, p=0.2476, n=5). C – Shows the average number of positively stained neurons per field of view for each group ($F_{(2,12)}$=1.788, p=0.2090,

n=5). D – Shows the average % area of positive staining per field of view for each group ($F_{(2,12)}$=0.7563, p=0.4905, n=5). White arrows show examples of positively stained neurons.

No significant changes could be found with CTCF analysis (p=0.2476), cell counts (p=0.2090) or % area with CCK1R positive staining (p=0.4905). This suggests no significant changes in CCK1R expression occur in the occipital area with age or CDS in the cat.

The parietal sections were analysed for CTCF, number of positively stained cells and % area with CCK1R staining (**Figure 65**).

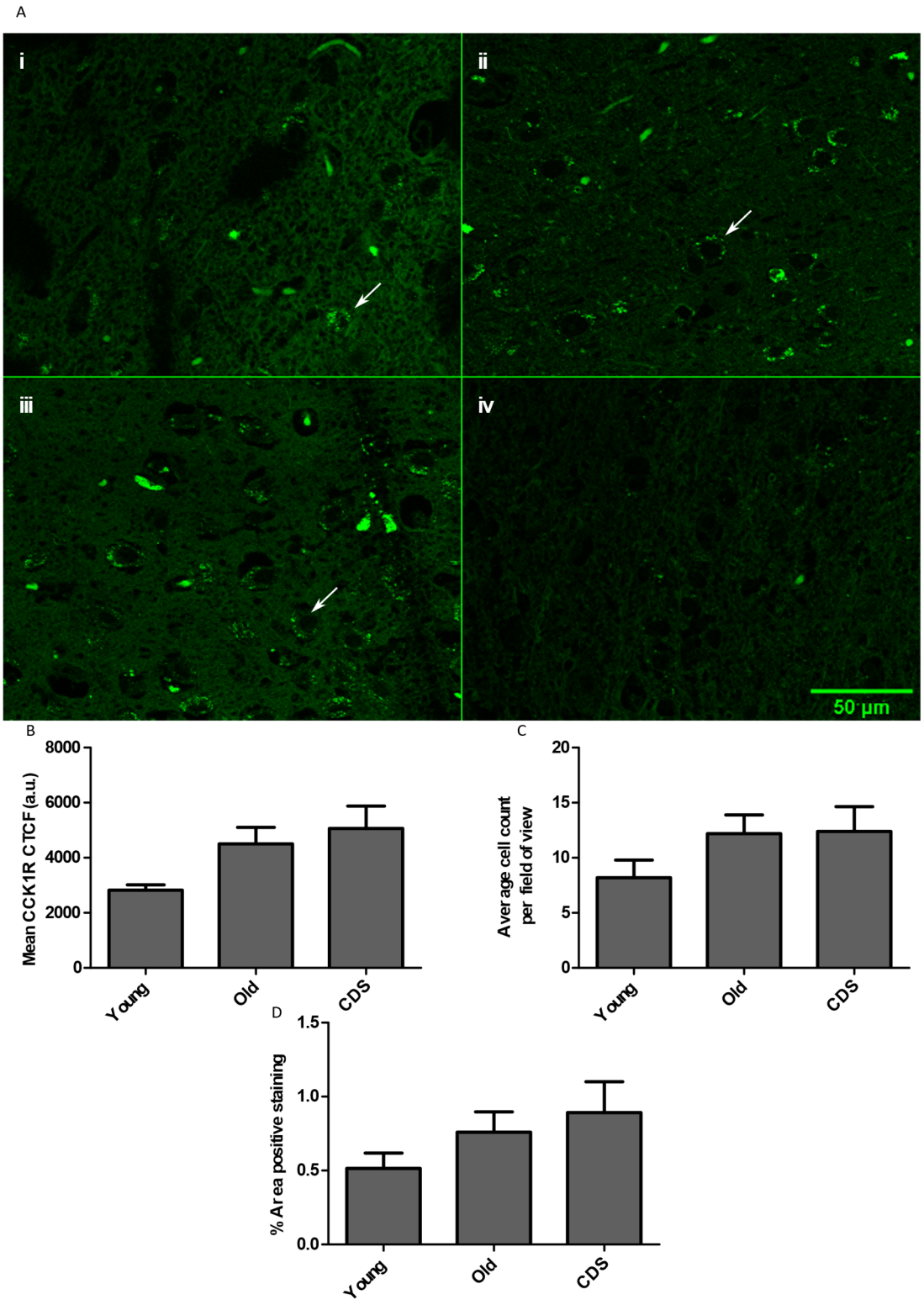

Figure 65. CCK1R staining in the parietal cat brain sections from young, old and CDS cats. Graphs show mean + SEM. A – Images show sections taken from occipital sections of young (i), old (ii), and confirmed diagnosis CDS (iii) cat brains stained with (i, ii, iii) or without (iv) CCK1R primary antibody and all containing a Dylight 488 secondary to detect receptor expression. B – Graph shows mean CCK1R CTCF as measured in positively stained neurons for each group ($F_{(2,12)}$=3.878, p=0.0502, n=5). C – Shows the average number of positively stained neurons per field of view for each group ($F_{(2,12)}$=1.613, p=0.2396, n=5). D – Shows the average % area of positive staining per field of view for each group ($F_{(2,12)}$=1.499, p=0.2624, n=5). White arrows show examples of positively stained neurons.

No significant differences were detected in any analysis (CTCF p=0.0502; cell counts p=0.2396; %area positive staining p=0.2624). No significant change in CCK1R expression occurred in the parietal section of cat brain with age or onset of CDS.

Finally, CCK1R staining was assessed in the rostral sections from the cat brains for CTCF, number of positively stained cells and %area of CCK1R staining (**Figure 66**).

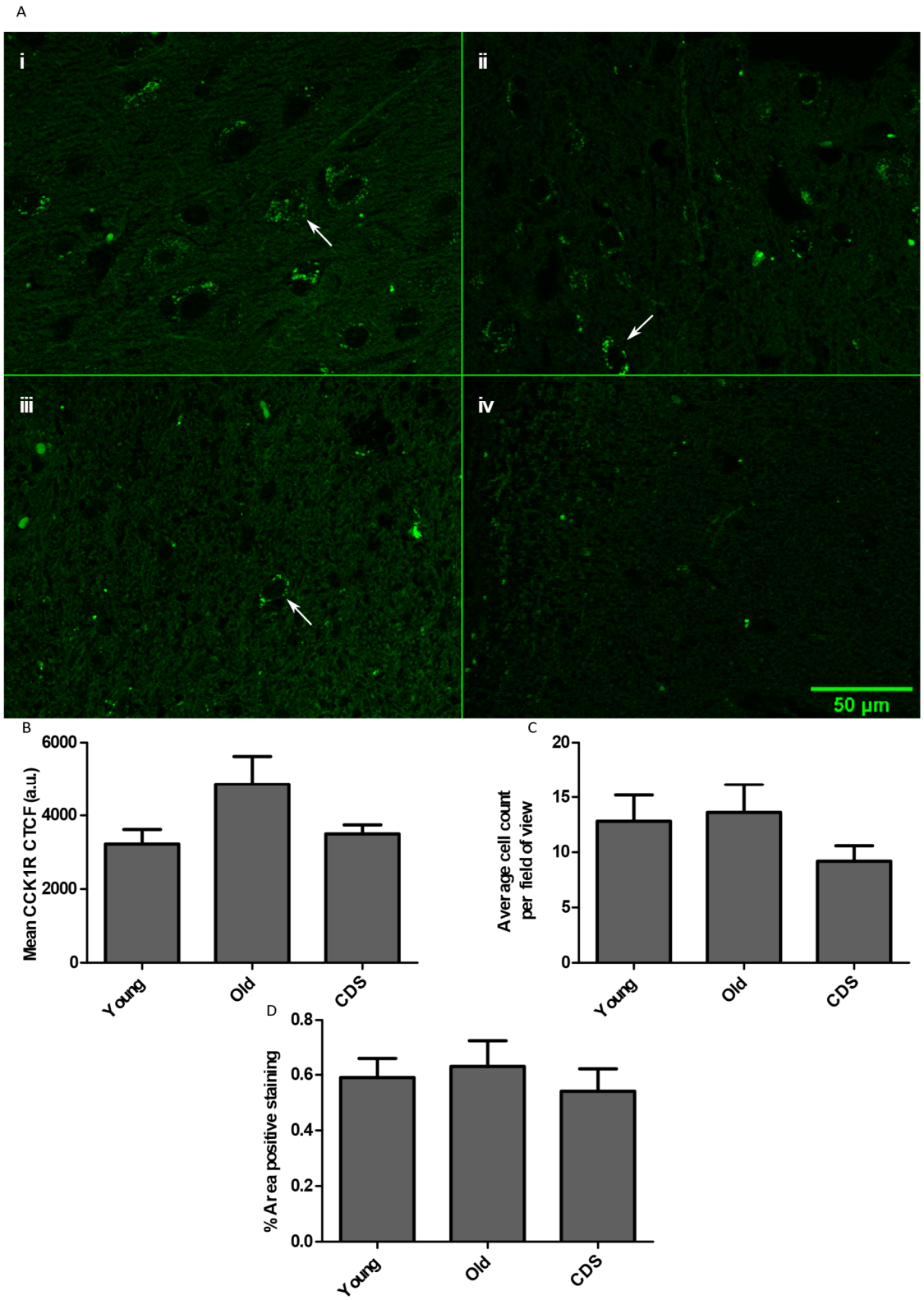

Figure 66. CCK1R staining in the rostral sections of young, old and CDS cat brains. Graphs show mean + SEM. A – Images show sections taken from rostral sections of young (i), old (ii), and confirmed diagnosis CDS (iii) cat brains stained with (i, ii, iii) or without (iv) CCK1R primary antibody and all containing a Dylight 488 secondary to detect receptor expression. B – Graph shows mean CCK1R CTCF as measured in positively stained neurons for each group ($H_{(2)}$=3.260, p=0.1959, n=5). C – Shows the average number of positively stained neurons per field of view for each group ($F_{(2,12)}$=1.157, p=0.3471,

n=5). D – Shows the average % area of positive staining per field of view for each group ($F_{(2,12)}$=0.2949, p=0.7499, n=5). White arrows show examples of positively stained neurons.

No significant changes in CCK1R expression occur in CTCF (p=0.1959), cell count (p=0.3471) or %area of CCK1R positive staining (p=0.7499) across age or with CDS.

Taken together this data demonstrates that ObR and CCK1R have regional changes in expression with age in the brain and further AD-like CDS in cats can differentially impact expression to old age. This suggests treatments relying on the activation of these receptors, such as A71623, leptin and leptin$_{116-130}$ may have different abilities to effect neurons based on brain region with age or disease.

7.7 Pilot study of Leptin, leptin$_{116-130}$ and A71623 treatments on aged neurons exposed to long-term low-dose Aβ.

AD is modelled in culture using Aβ treatments, but as yet a cell culture model which simulates the slow onset of AD using long-term treatments of Aβ to gradually induce toxicity has not been explored. As such long-term low-dose exposure of differentiated SHSY-5Y neurons to Aβ was evaluated for ability to induce a toxic effect. As CCK1R but not ObR receptor expression changes with age in human SHSY-5Y cells long-term treatments with A71623, leptin or leptin$_{116-130}$ were assessed for potential to overcome toxic effects of low-dose Aβ, toxicity and effectiveness of treatment, and differences between treatments over time.

Cells were differentiated and grown in mitotic inhibitor medium for one week before treatment, therefore at the time of treatment cells were at the one-week post-differentiation stage. For each experimental repeat three plates of cells were produced. Cell viability was analysed via CV and LDH assay. At seven-day intervals one of the triplicate plates were used for CV assay and medium was taken from all plates for LDH assay. Cells were treated with 2nM A71623, 1nM leptin or 1nM leptin$_{116-130}$ with or without 1μM Aβ and aged up to four weeks post-differentiation (3 weeks in treatment). Three experimental repeats were performed (Figure 67).

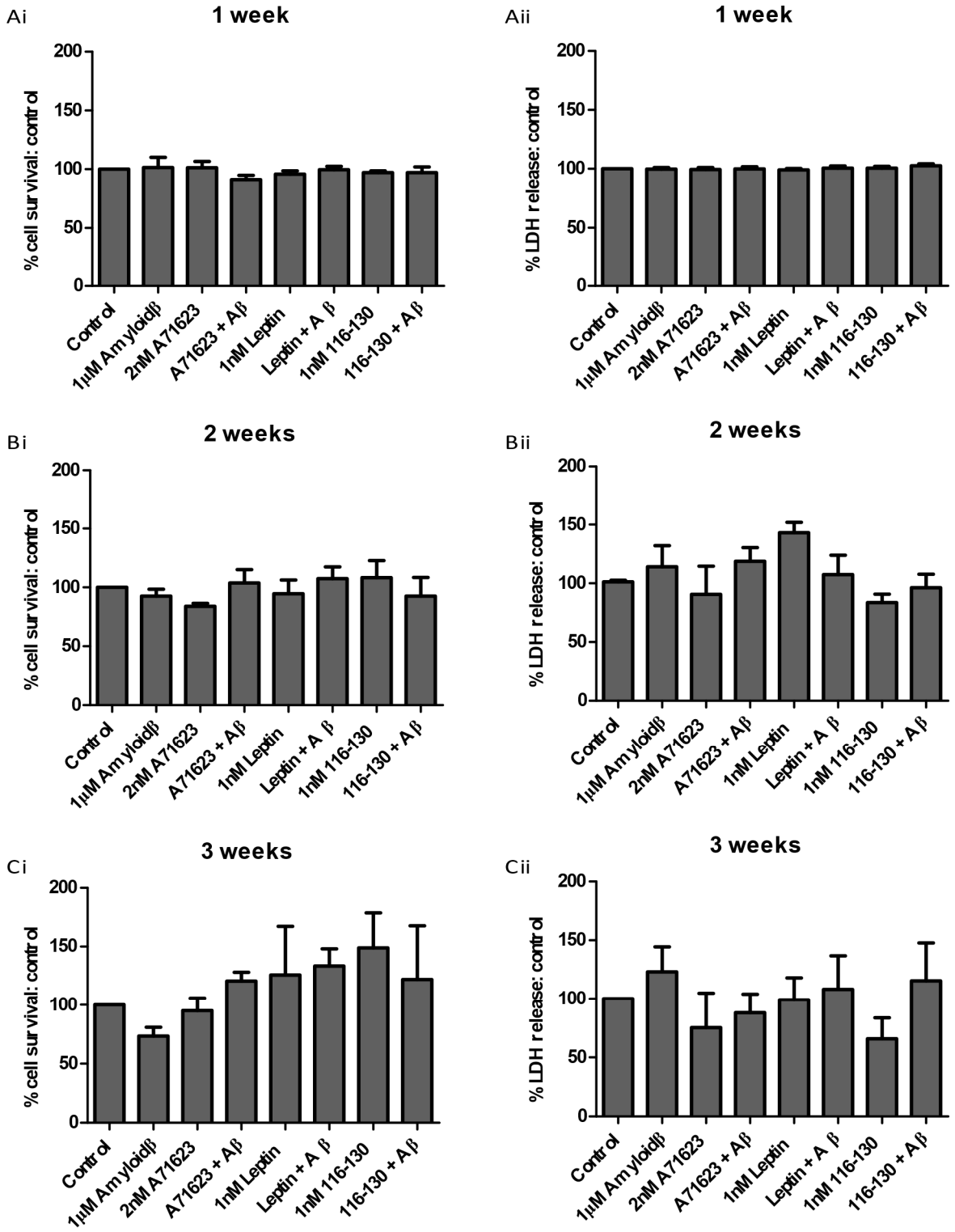

Figure 67. Neither low dose Aß, nor A71623, leptin or leptin$_{116-130}$ have significant effects on viability over 3 weeks of treatment in ageing SHSY-5Y neurons. Graphs show mean + SEM % cell survival as measured by CV assay (i) or % LDH release as measured by LDH assay (ii) relative to untreated control conditions. Viability was measured after one (A), two (B) and three weeks (C) in treatment. No significant effects were found (Ai $F_{(7,16)}$=0.5945, p=0.7515, n=3; Aii $F_{(7,24)}$=0.4985, p=0.8263, n=3; Bi $F_{(7,16)}$=0.6632, p=0.6997, n=3; Bii $F_{(7,16)}$=1.772, p=0.1624, n=3; Ci $H_{(7)}$=10.62, p=0.1561, n=3; Cii $F_{(7,16)}$=0.7420, p=0.6410, n=3).

No significant changes were seen in viability with 1μM Aβ treatment over 3 weeks or with any of the neuroprotectives: 2nM A71623, 1nM leptin or 1nM leptin$_{116-130}$ as measured by CV and LDH assays (n=3). As such Aβ could not induce cell death at this concentration over 3 weeks of treatment. However, treatment with A71623, leptin or leptin$_{116-130}$ did not induce toxicity either. As such whilst 1μM Aβ did not demonstrate the ability to slowly induce death with long-term treatment, A71623, leptin or leptin$_{116-130}$ treatments were not harmful to SHSY-5Y cells over 3 weeks of treatment.

7.8 Discussion

This chapter aimed to explore the synergistic relationship of CCK and leptin using co-treatment of CCK1R agonist, A71623, and leptin or leptin$_{116-130}$. Previous work has shown A71623, leptin and leptin$_{116-130}$ are neuroprotective (see 5.3 and 6.8) and CCK and leptin have a synergistic relationship (Barrachina *et al.*, 1997; Matson *et al.*, 1997; Peters *et al.*, 2005; Peters, Ritter and Simasko, 2006; Cano *et al.*, 2008; Heldsinger *et al.*, 2011; de Lartigue *et al.*, 2012). Therefore, low dose co-treatments were used to explore protective effects in undifferentiated and differentiated SHSY-5Y cells and potential signalling pathways were investigated. In addition, the use of co-treatments in modulation of AD biomarkers was assessed. Given that AD is an age-related disease and expression of receptors employed by A71623, leptin or leptin$_{116-130}$ may be affected by age an exploration of ObR and CCK1R across age using an ageing SHSY-5Y, rat cortex and a cat model of neurodegeneration was undertaken. Finally, the potential for 1μM Aβ to gradually induce cell death with long-term treatment was investigated and the potential for A71623, leptin or leptin$_{116-130}$ to be used over time on cells explored.

Co-treatment of undifferentiated SHSY-5Y cells using A71623 with leptin or leptin$_{116-130}$ demonstrated a protective effect in LDH but not CV assay against SGD. This suggests co-treatments were able to prevent cell membrane rupture in contrast to SGD treatment and as such produced a protective effect. Photomicrographs were employed to visually compare treatment conditions and whilst SGD condition demonstrated fewer cells other conditions were similar. Whilst these results initially appear contrasting it was suggested this result could be produced if co-treatments were

increasing cell mitosis, as cell adherence decreases during mitosis (Marchesi *et al.*, 2014). From DAPI stained images changes in morphology of the cells were clear as such cell counts of normal, mitotic and apoptotic cells relative to total cell number per image were employed. Results showed all conditions had significantly lower normal cells compared to control, but co-treatments had significantly higher proportion of mitotic cells and all treatments except A71623 with $leptin_{116\text{-}130}$ had a significantly higher proportion of apoptotic cells compared to the control condition. These results suggest while some cell death is being induced in these cells, co-treatments also increased mitosis which explains the CV result.

CCK and leptin have both previously been linked to mitosis. In the rat pancreas CCK-8 increased growth by increasing mitosis and A71623 showed a similar effect whereas a CCK2R agonist (SNF-8815) demonstrated no effect (Povoski *et al.*, 1994). This shows CCK can induce mitosis and it is mediated via CCK1R. Further, leptin increases cell proliferation when injected into pre-metamorphic Xenopus tadpole brains (Bender, Sifuentes and Denver, 2017). It is also highlighted in the process of brain development in rodents where leptin increased proliferation and differentiation (Udagawa *et al.*, 2006) and ob/ob leptin deficient mice which have smaller brain weight and cortical volume. Administration of leptin to these mice during development corrected this reduction (Steppan and Swick, 1999). However, leptin could not increase proliferation in the human microvascular endothelial cell line (Álvarez *et al.*, 2012). This difference may be due to cell type or species, however, given neither low-dose treatment of A71623, leptin or $leptin_{116\text{-}130}$ significantly increased mitosis alone but the effect occurred with both co-treatments this lends credence to a synergistic interaction which can mediate mitosis.

As co-treatments of A71623 with leptin or $leptin_{116\text{-}130}$ reduced membrane rupture in undifferentiated cells, neuroprotection from copper toxicity was examined in differentiated SHSY-5Y cells. Whilst the CV assay showed no protective effects of co-treatment, the LDH data shows both 0.01nM leptin alone and co-treatment of A71623 and $leptin_{116\text{-}130}$ had reduced LDH release compared to the copper treated condition. This data suggests 0.01nM leptin can produce a protective effect and

as such may not have been the optimal concentration to use in this experiment. However, when combined with A71623 this effect disappeared. Furthermore, the opposite trend occurs with leptin$_{116-130}$ where neither 0.08nM A71623 nor 0.01nM leptin$_{116-130}$ were able to produce a protective effect but in combination LDH was reduced. This is in agreement with previous work which suggest a synergistic relationship between CCK and leptin (Barrachina *et al.*, 1997; Matson *et al.*, 1997; Peters, Ritter and Simasko, 2006) as combination therapy of A71623 and leptin did show a differential effect to leptin alone. However, it implies CCK1R agonism was able to reverse positive effects of 0.01nM leptin in this case. Furthermore, this shows a difference in action of leptin and leptin$_{116-130}$ as 0.01nM leptin could reduce LDH but 0.01nM leptin$_{116-130}$ could not. Further co-treatment of 0.08nM A71623 and 0.01nM leptin$_{116-130}$ could reduce LDH release but 0.08nM A71623 with 0.01nM leptin could not. This may imply that in fully differentiated neurons the synergistic protective actions of CCK and leptin are reliant on the actions of amino acids 116-130 but via CCK1R signalling interaction with full-length leptin has other effects.

An explanation for this may lie in the structural properties of leptin and leptin$_{116-130}$. Leptin has structural similarity to other cytokines including granulocyte colony-stimulating factor (G-CSF), leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and human growth hormone. Further the extracellular domain of ObR also shows similarities to glycoprotein gp130 signal transducing subunits of interleukin-6 (IL-6) receptor, G-CSF receptor and LIF receptor and as such is hypothesised to have similar modes of binding (Zhang *et al.*, 1997; Peelman *et al.*, 2004). These cytokines typically have 3 binding sites and when structures of mouse leptin, human leptin, human CNTF, human IL-6, bovine G-CSF and murine LIF were superimposed binding site alignment was found. As such residues within these binding sites in leptin were mutated and their effects on signalling and ObR binding studied. Mutations at amino acids 34/35, 41, 115, 117, 122, 138/139/142 caused decreased maximal signalling. Clustering of mutants with greatly decreased maximum signalling activity was seen around inactive 120/121. Mutations in amino acids 9/12, 15, 16, 20, 75, 82/85, 86 of leptin inhibited binding to the CRD of ObR. This data shows mutating leptin at amino acids 120/121 still allows binding to the CRD

but does not increase STAT3 signalling therefore cannot activate the receptor (Peelman *et al.*, 2004). Therefore, amino acids within leptin$_{116\text{-}130}$ are important for inducing signalling but not binding to ObR. Due to the structural similarity of other receptors extracellular domains and this evidence showing leptin$_{116\text{-}130}$ does not contain domains suggested to be important for binding of full-length leptin this may mean leptin$_{116\text{-}130}$ is able to activate another receptor. One candidate for this may be the IL-6 receptor. IL-6 has been shown to use STAT3 (Heinrich *et al.*, 1998) and Akt signalling (Nishikai-Yan Shen *et al.*, 2017) which is compatible with my earlier findings that leptin$_{116\text{-}130}$ signals through these pathways (See 5.4). Further IL-6 has been linked to CCK as addition of IL-6 to pancreatic acinar cells caused 2x increase in binding capacity of CCK1R and CCK2R (Viguerie *et al.*, 1994). Therefore the abilities of A71623 and leptin$_{116\text{-}130}$ co-treatment to prevent membrane rupture may be due to leptin$_{116\text{-}130}$ signalling via IL-6 receptor leading to increased binding affinity of A71623 to CCK1R rather than via a synergistic action dependent on ObR.

Previous work states the synergistic effect of leptin and CCK on food intake, through CCK1R, is mediated by STAT3 and PI3K pathways (Heldsinger *et al.*, 2011), however my exploration of these pathways demonstrated that whilst A71623, leptin and leptin$_{116\text{-}130}$ increased pSTAT3 levels, no further increase was found with co-treatments, and no significant changes in pAkt compared to control. I have shown leptin and leptin$_{116\text{-}130}$ can signal through these pathways (see 5.4), A71623 can signal via the STAT3 pathway (See 6.9) and previous work has suggested PI3K/Akt is downstream of the CCK1R signalling pathway (Zhou *et al.*, 2014). One explanation for the differences in my findings may be the time points at which cells were used for examination of signalling. In the leptin$_{116\text{-}130}$ experiment (see 5.4) signalling was examined after just 3-hour treatments, however in the A71623 and co-treatments signalling was examined after the full length of treatment used in the viability assays, 96h, to understand how signalling persisted throughout treatment. It is possible signalling through the PI3K pathway is an initial response and longer-term signalling is via the STAT3 pathway. Previous work

stimulated rat nodose ganglion for just 10minutes with 1nM leptin and 1nM CCK-8 and found an additive increase in pSTAT3 relative to separate treatments, further this was inhibited by PI3K inhibitors LY294002 and wortmannin. Co-treatment also saw a significant increase in pPI3K after 10-minute treatment. Together this evidence led to the conclusion PI3K was pivotal to the synergistic interaction between CCK and leptin (Heldsinger *et al.*, 2011). As such another explanation for my results is a PI3K pathway is being employed in co-treatments which does not involve signalling via Akt. As previously mentioned (see 6.12) a direct link between PI3K and STAT3 has been identified in the PI3K/BMX/STAT3 pathway (Vogt and Hart, 2011), upregulation of which may generate anti-apoptotic effects (Peng *et al.*, 2016). As such combined treatments of A71623 and leptin or leptin$_{116\text{-}130}$ may rely on this pathway. The result showing no additive increase in pSTAT3 with co-treatments may indicate that an additional signalling pathway is activated rather than additive activation occurs with co-treatments.

As A71623, leptin and leptin$_{116\text{-}130}$ suggested trends in modulation of AD biomarkers and combined treatments of A71623 with leptin$_{116\text{-}130}$ has some protective abilities in a neuronal phenotype, the effects of co-treatments were explored. As discussed, (see 5.9) 1μM Aβ was unable to reliably upregulate the biomarkers chosen using this experimental design, therefore further refinement is required. However, trends in the data may be worth assessing. As data from individual treatments have already been discussed (see 5.9 and 6.12) this discussion will focus on the co-treatments.

A71623 significantly reduced p-tau increase cause by Aβ previously (see 6.12) but co-treatments with leptin or leptin$_{116\text{-}130}$ results in p-tau level similar to 1μM Aβ treatment. Leptin has previously been suggested to modulate p-tau (Greco *et al.*, 2009; Li *et al.*, 2016) and leptin impaired OLEFT rats show increased p-tau from 42 weeks old (Jung *et al.*, 2013). However, these same rats also lack CCK1R (Jung *et al.*, 2013) as such it is possible p-tau accumulation is due to a lack of CCK1R rather than leptin. Leptin's suggested abilities to modulate p-tau were dependent on increased AMPK signalling (Greco *et al.*, 2009) however AMPK activation has also been linked to increased

phosphorylation of tau (Domise *et al.*, 2016) and co-administration of leptin and CCK decreases AMPK activation in the hypothalamus (Akieda-Asai, Poleni and Date, 2014). Conversely CCK-8 alone activates leptin signalling pathways to increase AMPK phosphorylation (Merino *et al.*, 2008) and leptin can inhibit AMPK (Minokoshi *et al.*, 2004). As such this evidence creates a complicated relationship between CCK, leptin and AMPK activation. Therefore, co-treatments failing to ameliorate p-tau increase is indicative of a concentration-sensitive relationship, whereby when CCK1R activation can ameliorate tau phosphorylation alone the addition of leptin overrides this effect, possibly by switching AMPK signalling off. Alternatively, it may be the concentration of leptin and $leptin_{116-130}$ used is such that it causes changes in AMPK signalling which prevent its modulation of p-tau and CCK1R activation cannot overcome this as it affects the same pathway.

In this experiment there were no pronounced changes in ABAD across treatments of leptin, $leptin_{116-130,}$ A71623 or co-treatments. However previously A71623 treatment was close to significantly decreasing ABAD in the presence of Aβ (see 6.12). Little work has been done on the relationship between leptin and ABAD (See 5.9), and given its link to the mitochondria (Holmström *et al.*, 2013; Blanquer-Rosselló *et al.*, 2015; Munusamy *et al.*, 2015) this is an interesting avenue for future work. CCK has been linked to the ABAD starvation response through ketones (see 6.12)(Du Yan *et al.*, 2000; Chearskul *et al.*, 2008) and leptin has also been linked to ketones as it upregulates gene expression of acetoacetyl-CoA synthetase which uses ketone bodies to synthesise lipids (Narishima *et al.*, 2011). However, it is unlikely a starvation-related response would occur in these cultures, given the plentiful energy supply in culture medium, as such these mechanisms would not be employed and may explain why little change in ABAD was seen.

Little change is seen in APP with 1μM Aβ treatment. However, t-tests show both co-treatments in the presence of Aβ have significantly lower APP that 1μM Aβ treatment alone (A71623 with leptin p=0.0115, A71623 with $leptin_{116-130}$ p=0.0173). Whilst leptin previously hinted at an ability to decrease APP, neither A71623 nor $leptin_{116-130}$ did. This significant difference with co-treatment may suggest a

synergistic effect. Leptin has been shown to attenuate NF-κB (Marwarha *et al.*, 2014) which in turn can increase APP (Chami *et al.*, 2012), but it was suggested CCK was downstream of APP signalling (Hansen, Rehfeld and Nielsen, 2004; Kirouac *et al.*, 2017). Therefore, in this case it may be CCK1R agonism is enhancing the effects of leptin on APP rather than having a direct impact on APP itself.

For endophilin 1 all treatments increased expression of this biomarker. In the leptin and CCK insensitive Zucker rat endophilin 1 levels are increased (Doherty *et al.*, 2013) as such it is unexpected leptin or A71623 would increase endophilin 1. It was previously suggested leptins role in endophilin 1 increase here may be related to vesicle release and endocytosis (see 5.9). Whilst a direct link between CCK1R activation and endophilin 1 has yet to be determined, CCK has been linked in the pancreas to SNAP-23 a component in vesicle transport machinery (Huang *et al.*, 2001). Further the brain SNAP-23 homologue SNAP-25 interacts with endophilin 1 through SNIP (Chin *et al.*, 2000; Yang *et al.*, 2015) (see 6.12). Therefore, CCK may indirectly increase endophilin 1 through a relationship with SNAP-25. As such leptin and CCK1R agonism may be working via separate mechanism to increase synaptic activity via endophilin 1.

Presenilin 1 was not increased in the cultures by Aβ, but co-treatment of A71623 with leptin in the presence of Aβ may show decreased presenilin 1 levels (t-test p=0.1336) but A71623 with $leptin_{116\text{-}130}$ in the presence of Aβ show no difference from 1μM Aβ alone. Leptin treatment has been shown to decrease presenilin 1 levels (Niedowicz *et al.*, 2013) and we suggested a potential mechanism by which CCK could moderate presenilin 1 via filamin (see 6.12) and whilst alone these did not result in decreased presenilin 1, these effects may be separate but complimentary.

Trends in the biomarkers suggest co-treatments of A71623 with leptin or $leptin_{116\text{-}130}$ can show differing effects demonstrating some synergistic actions are related to leptin's effects outside of the 116-130 amino acids. At the concentrations used co-treatments override A71623's ability to modulate tau phosphorylation. APP levels were decreased in co-treatments and this appeared to due to a synergistic action of CCK1R agonism enhancing actions of leptin and $leptin_{116\text{-}130}$. Endophilin 1 was

previously increased by A71623, leptin and leptin$_{116-130}$ alone, but co-treatments did not show an additive effect. However, A71623 with leptin treatment decreased presenilin 1 but A71623 with leptin$_{116-130}$ saw levels similar to control and Aβ conditions. As such there may be a role for leptin in this relationship which can be exacerbated by CCK1R agonism but is reliant on actions of leptin not controlled by amino acids 116-130. In order to confirm these trends further optimisation of this technique is required.

To assess changes in the leptin and cholecystokinin receptor with age and hence how a long-term treatment might be affected in an age-related disease, changes in ObR and CCK1R were measured in an ageing SHSY-5Y model. Further to compare these changes with ageing *in vivo* and with disease states the rat and feline models were employed as well as cats with naturally occurring CDS. To explore these changes ELISA and immuno-fluorescent imaging techniques were employed.

By ELISA technique both the ageing SHSY-5Y model and rat cortex extracts showed no significant changes in ObR expression with age. Similarly, it has previously been demonstrated ObRb expression does not change with age or gender, but ObRa decreased with age, a change which was much larger in females than males, however, this change in receptors was measured from peripheral blood mononuclear cells (Roszkowska-Gancarz *et al.*, 2015) so may not be an accurate representation of changes in the brain. On the other hand, the antibody in my experiments detects the extracellular component of ObR, as such is unable to measure changes in specific isoforms.

Similarly, to SHSY-5Y cells and rat cortex, the cat brain showed no significant changes in expression in Purkinje and granule cells of the cerebellum or in the parietal lobe, with age or onset of CDS. In the cerebellum leptin promotes Purkinje cell survival and has no effect on granule cells in mice (Oldreive, Harvey and Doherty, 2008). Further, With AD in humans the cerebellum is affected during the late stages of the disease and is relatively spared from Purkinje loss (Calderon-Garcidueñas and Duyckaerts, 2018). Therefore, no significant change in ObR in the cerebellum with CDS in cats may

reflect its AD-like nature. However, in AD medial and posterior areas of the parietal lobe are preferentially affected by neuronal loss (Jacobs *et al.*, 2012). Therefore, we might expect some decrease in number of positively stained neurons in CDS cats due to neuronal loss. It may be the imaging technique employed was not specific enough to identify precise regions impacted by CDS within this area. Whole section imaging would allow for a more complete comparison here. In contrast the occipital and rostral sections do show significant changes. In the occipital lobe an increase in the percent area of positive staining in old compared to young cats was seen and no difference between old and CDS cats. In the rostral section cells had greater ObR expression per cell in old cats compared to both young and CDS cats and greater percent area ObR staining than CDS cats with no difference between young and CDS. These results demonstrate changes with both age and CDS. Both sections demonstrate greater area of ObR staining in old cats, which may be indicative of either grater extracellular ObR expression or larger cells. From the representative images it is apparent that cells are larger in old and CDS cat's occipital lobe, however in the rostral section cats appear to have more extracellular staining. In the occipital lobe, this is likely a result of maturation and learning. The visual cortex lies within the occipital lobe thus ObR expression here may be due to leptins satiety role and therefore involved in food recognition (Thanos *et al.*, 2008). The visual cortex also has a role in memory whereby in the short-term it can store information about current events (van Kerkoerle, Self and Roelfsema, 2017) but also must feed into and access a long-term store for recognition of objects (Cooke *et al.*, 2015; Takeda, 2018). This is the case with cats as evidenced by one-trial visual recognition task whereby cats presented with a novel object indicating the location of a food reward could recall this learned visual association after a delayed recall time of 10minutes (Okujava *et al.*, 2005). It is possible therefore that the increase in leptin receptor expression with age in the occipital lobe of the cats is related to an increase in use and recognition memory over time. The lack of significance in the CDS group relative to the young group may indicate the variation in disease level. For instance, it may be that receptor levels will decline in this area with CDS but at an intermediate disease stage. Conversely, CDS did show a significant impact on the rostral sections whereby ObR expression was decreased and indistinguishable

from young cats. Whilst this may be a sign of neuronal atrophy in this area, the data suggests no changes in the number of cells with any ObR expression across the three groups suggesting this shows a loss of ObR expression rather than a decrease in cell number *per se*.

In contrast to ObR, ageing SHSY-5Y cells had significantly decreased CCK1R expression at 3- and 4-weeks post-differentiation both compared to undifferentiated and differentiated SHSY-5Y cells, but no significant change was found in CCK1R expression with age in the rat cortex. Previous work has shown CCK injected both IP and intracerebroventricularly (ICV) are impacted by age in male Wistar rats, with IP injections showing strong anorexic effects at both young (4-6months) and old (18-24months) but little impact in middle aged rats (12months) and further ICV injections showing gradual decline in effect which disappears by 24months of age (Balaskó *et al.*, 2013). These findings are likely to indicate either a change in receptor expression or the development of CCK resistance with age in the brain.

Similarly, to the rat cortex no significant changes in CCK1R expression were seen in occipital, parietal or rostral sections of the cat brain. Both rostral (Wang, Yaksh and Go, 1983) and visual cortex (Demeulemeester, Vandesande and Orban, 1985; Freund *et al.*, 1986; Meyer and Wahle, 1988) have CCK immunoreactive neurons in cats. In the visual cortex these are unaffected by age in cats (Meyer and Wahle, 1988) or by AD in humans (Gabriel *et al.*, 1996) which may agree with our findings that show no receptor change in the occipital area. Little work has been done on CCK in the rostral and parietal cortices of the cat with age or CDS. Post-mortem studies in AD human brains has shown decreases in CCK in some areas of the cerebral cortex though these were heavily influenced by post-mortem delay (Perry *et al.*, 1981; Rossor *et al.*, 1981; Ferrier *et al.*, 1983; Mazurek and Beal, 1991) and in mouse AD models little change was seen in CCK in this area and due to perfusion techniques had minimal post-mortem delay (Diez *et al.*, 2003). Together this data indicates little impact of age or CDS on CCK1R expression throughout the cat cortex.

However, a significant change with age was found in Purkinje cells of the cerebellum, whereby significantly reduced CCK1R was seen per cell in old cats compared to young cats. No significant difference was seen between young and CDS cats. CCK binding sites have been identified in the human cerebellum, particularly in granular layer and Purkinje cells (Goldman *et al.*, 1987), and expression here is highest in humans, rats and pigs at the foetal stage after which it declines (Rehfeld *et al.*, 1992). It is possible therefore that the decreased CCK1R staining in old cat Purkinje cells is due to maturation and the increase in expression with CDS is an indication of dedifferentiation. CCK is important in regeneration of the pancreas following injury, a processes known to be linked to acinar cell dedifferentiation (Guo *et al.*, 2012). Further rats lacking CCK1R show decreased regenerative abilities in pancreatic tissue (Miyasaka *et al.*, 1997, 1998). Whilst Purkinje cells do not dedifferentiate under normal conditions, the inhibition of retinoblastoma protein (Rb1) causes them to re-enter the cell cycle (Feddersen *et al.*, 1995). Rb1 has been linked to AD though the nature of this relationship remains unclear (Muñoz *et al.*, 2005; Absalon *et al.*, 2013; Pavlopoulos *et al.*, 2013; Huang, Lee and Chen, 2014). Therefore, it is possible in CDS a decline in Rb1 causes dedifferentiation of Purkinje cells which increases CCK1R expression indicative of their immature state.

Altogether this data highlighted changes in ObR and CCK1R expression as region specific with age, disease and species. These intricate patterns demonstrate how the selection of models and regions of interest when studying both the ageing process and neurodegenerative diseases is vital to understanding and abilities to treat changes. Further, this emphasises the need to understand natural changes with age in the brain in order to be able to identify changes which are a part of ageing and those which are impacted by disease. These changes are vital to our understanding of how effective treatments for age-related disease, such as AD would be. To further study this within a laboratory setting models which mirror disease progression are required. Further development of existing models will allow for better translation from lab to clinical trials. Therefore, the final investigation undertaken as part of this thesis was to identify the potential for long-term low-dose Aβ as a model and test the effects of our treatments with age.

No significant changes in viability were detected over these experiments, however, given this was a pilot study, a low number of experimental repeats were obtained and variation in cell survival was much greater with age than in younger cultures which may explain this. Whilst changes seen after two weeks in culture are small or highly variable some trends may be evident after 3 weeks post-differentiation. This data suggests Aβ treated cells may have decreased viability and whilst there remains high variability in the leptin and leptin$_{116\text{-}130}$ data, A71623 treated cells have much lower deviation and hint at a protective effect. As such this model may provide useful understanding of long-term use of treatments on neurons but requires further refinement. This experiment provided novel insight into how SHSY-5Y neurons respond to low-dose Aβ treatment over time, as no previous research has investigated this the experimenter had no knowledge of the toxicity this treatment would have. Therefore, unlike viability assays used throughout this thesis which employed an exclusion criterion for analysis, all data was analysed to elucidate if 1μM Aβ could induce cell death. This may account for some of the increased variability in viability data as in other experiments incidences where the toxic condition did not reduce CV readings by 15% or increase LDH readings by 15% were excluded. With greater experimental repeats it will become clear if this exclusion criteria can be applied and as such only experiments where 1μM Aβ is deemed to have sufficiently induced cell death would be included, thus a clear reflection of A71623, leptin and leptin$_{116\text{-}130}$'s abilities to protect against this could be elucidated. Power analysis under these criteria suggests an n of 12 would be required for statistical power. As such application of a criteria to the Aβ condition to be considered cell death and greater experimental repeats are needed to confirm the trends in this data.

In this chapter it was shown that A71623 with leptin$_{116\text{-}130}$ demonstrate promise as a co-treatment, whereas A71623 with leptin appears not to have a neuroprotective effect in the model used. This demonstrates a divide in the actions of leptin and leptin$_{116\text{-}130}$, especially in the context of a synergistic relationship with CCK. It suggests that amino acids 116-130 are essential for the

neuroprotective nature of the combined effects and deeper exploration of the action of leptin$_{16\text{-}130}$ is required to understand this difference. A demonstration that expression of these compounds' receptors change with both age and disease may be highly regional and cell-type specific. As such individual treatments of CCK1R agonism or leptin may be able to target specific sites but changes in their receptors may prevent a whole brain effective treatment. This lends support to the idea of a multi-faceted combined treatment. This data also highlights how different laboratory models of age and disease can vary in factors important for treatment efficacy, such as receptor expression. Thus, models most reflective of the target species, i.e. humans, must be expanded upon. Whilst the pilot study of using low-dose Aβ to induce toxicity in the human SHSY-5Y neurons did not produce significant effects, it demonstrated promise as a potential method for studying longer-term effects of treatments.

Therefore, this chapter has demonstrated that through their synergistic relationships CCK1R agonism and leptin may provide an effective treatment against AD, but the neuroprotective nature of this relationship may be most effective when only amino acids in the 116-130 range of the leptin hormone are employed.

8 DISCUSSION AND CONCLUSION

This thesis sought to highlight the importance of the endocrine system in ageing and how interventions which focus on the natural abilities of hormones can provide new insights into potential disease-modifying treatments for neurodegenerative diseases such as Alzheimer's disease. In order to show this, I focussed on two hormones. Leptin has previously demonstrated beneficial effects within AD relevant conditions (Doherty *et al.*, 2013; Pedrós *et al.*, 2015) but also demonstrates significant drawbacks as a treatment itself (García-Robles, Segura-Ortega and Fafutis-Morris, 2013; Alvarez *et al.*, 2014; Chang *et al.*, 2015; Denroche *et al.*, 2015; Younger *et al.*, 2016; Rodrigo *et al.*, 2017). Building on work which suggested peptide fragments could be used as viable therapeutics (Lipinski, 2016; Santos, Ganesan and Emery, 2016) and work with previous leptin fragments which shows residues between amino acids 116-130 are essential for leptin's weight reducing effects (Grasso *et al.*, 1997; Rozhavskaya-Arena *et al.*, 2000), the fragment leptin$_{116\text{-}130}$ was assessed as a potential treatment for AD. I aimed to investigate whether leptin$_{116\text{-}130}$ had equivalent neuroprotective actions to full-length leptin *in vivo* and *in vitro*. Further, a pilot assessment of hexamers from within leptin$_{116\text{-}130}$ were tested for their potential neuroprotective effects as they would further lend to modifications necessary to become an oral therapeutic. As discussed, (see **5.1**) orally available therapeutics usually confer to the rule of five (Lipinski *et al.*, 2001; Lipinski, 2004) and native peptide hormones are susceptible to metabolism (Fotherby, 1996). Whilst hexamers from leptin$_{116\text{-}130}$ address the size issue, modifications to overcome degradation in the gut are necessary and these hexamers have been identified as susceptible to modification. Methods for overcoming degradation include; using proteolytic inhibitors or passive absorbance enhancers and chemical and amino acid backbone modifications (Truong-Le, Lovalenti and Abdul-Fattah, 2015), encapsulation in polymeric microspheres (Alcalá-Alcalá *et al.*, 2013; Du *et al.*, 2013), molecular stapling using hydrocarbon bonds to stabilise peptide structure (Chapuis *et al.*, 2012), and cyclization (Yao *et al.*, 2018). This latter strategy also improves stability and cell permeability (Reichart, Horn and Neundorf, 2016; Rubin and Qvit, 2018) and has already been used for other peptide

hormones, oxytocin and vasopressin (Deyle, Kong and Heinis, 2017). As such this demonstrates a realistic potential for the use of these compounds as therapeutics.

The first results chapter (**5**) demonstrated that leptin$_{116\text{-}130}$ mirrors the neuroprotective abilities of full-length human leptin and can act as a enhancer of episodic memory, the earliest form of memory affected by AD. Results also show even smaller fragments from within the 116-130 amino acid sequence can demonstrate neuroprotective effects. This lends credence to fragments of leptin as therapeutics in AD. This work is important because it reveals a clear drug target for AD treatment. Further, as leptin has previously been shown to have great promise in providing a disease-modifying treatment in AD but is unusable due to its side effects the fact that just a fragment of the molecule can mirror its most vital actions shows off-target effects could be overcome.

The most important next step for this project is to optimise the non-lethal method of inducing AD-biomarkers by Aβ. The inconsistency of results from the current methodology resulted in low experimental numbers and thus inconclusive results as to the impact of leptin and leptin$_{116\text{-}130}$ on these biomarkers. This is especially crucial in the case of results conflicting those in published literature, such as with endophilin 1. Other studies using the same concentration of Aβ either used much shorter (Costa *et al.*, 2012; Doherty *et al.*, 2013; Gilson *et al.*, 2015) or longer treatment periods (Calvo-Rodríguez *et al.*, 2017). This differing length of treatment to induce biomarkers may be an indication of different cell-type sensitivity to 1μM Aβ as none of these studies used SHSY-5Y cells, but it may also indicate some compensatory mechanism in cells triggered by this treatment. For example, it may be initial dosage of Aβ begins to induce biomarkers, but cells are able to recover from this, hence short treatment periods see changes in biomarkers which can be returned to normal by cultures natural defences therefore mid-length treatment time produces inconsistent changes. However, as treatment continues Aβ build-up overcomes cellular defences and biomarker levels increase again. Thus, longer treatments also demonstrate changes. Therefore, I would suggest length of treatment should be explored both to understand optimal treatment for these experiments but also to elucidate this pattern of biomarker

change. This could be tested by ICC or ELISA assay to measure p-tau expression in 1μM Aβ cultures relative to untreated cultures at 24h, 48h, 72h, 96h and 7 days post-treatment. Once able to reliably induce biomarker expression in these cultures' experimentation with leptin and leptin$_{116-130}$ should be repeated. Further exploration of this data by western blotting alongside ELISA may give more detail about protein expression or use of ICC could indicate changes in localisation.

An important line of enquiry is the ability of leptin$_{116-130}$ to activate ObR. One published experiment has demonstrated leptin$_{116-130}$ was able to induce its weight reducing effects in the absence of ObRb (Grasso *et al.*, 1999). Work has also suggested that leptin does not require ObR to cross the BBB (Gonzalez-Carter *et al.*, 2016) and that LRP-2 is employed in leptin crossing into the CSF (Dietrich *et al.*, 2008). As such the ability of leptin to bind to different receptors and if leptin$_{116-130}$ employs a different receptor to mediate its actions should be explored. Initial work should confirm if leptin$_{116-130}$'s neuroprotective abilities are independent of ObR. This can be tested using ObR inhibitors such as antibody 9F8 (Gonzalez-Carter *et al.*, 2016) or by knocking-out the ObR receptor in the SHSY-5Y cell line and running viability assays as previously with optimal concentrations of leptin and leptin$_{116-130}$. Receptor binding assays could also be run to directly measure binding of leptin and leptin$_{116-130}$ to ObR, as well as to other potential targets such as LRP-2 (Weissman *et al.*, 2007). Further, work has suggested ObR isoforms other that ObRb may have signalling capabilities (Fei *et al.*, 1997; Belouzard and Rouillé, 2006; Séron *et al.*, 2011) and similarities between extracellular binding domains of other cytokines e.g. IL-6 (Zhang *et al.*, 1997; Peelman *et al.*, 2004). As such how leptin$_{116-130}$ interacts with the different isoforms of ObR and other cytokines should be explored. This will add to my current work on leptin$_{116-130}$'s neuroprotective effects and may elucidate other signalling pathways employed by this compound.

Further work should also include AD animal models both in the exploration of leptin$_{116-130}$'s abilities to enhance memory and attenuate pathology, but also to ascertain differences in long-term side effects between leptin and leptin$_{116-130}$. As I have shown leptin$_{116-130}$ has neuroprotective and cognitive enhancing effects, its potential as a treatment in AD can be further explored by employing animal

models. Some of the most common models used for exploration of AD are the APP/PS1 and Tg2576 mouse models, both of which have been employed to investigate the effects of full-length leptin previously. In these models leptin has been shown to attenuate Aβ-induced neurodegeneration (Pérez-González *et al.*, 2011), decrease p-tau and improve memory (Greco *et al.*, 2010; Pérez-González *et al.*, 2014). Leptin$_{116-130}$ has demonstrated capability for all these traits *in vitro* and is comparable to leptin as such it would be expected it would match these abilities of leptin *in vivo*. Mice do not naturally develop AD; hence the disease must be induced through genetic mutation. The APP/PS1 model demonstrates deficits in hippocampal function, Aβ deposition from 3 months of age, abnormal tau phosphorylation (but no NFTs) and neuronal loss at late stages (22-24 months) (Porquet *et al.*, 2015; Gelman *et al.*, 2018). The Tg2576 model demonstrates learning and memory impairment, amyloid plaques, neuroinflammation, loss of noradrenergic but not cholinergic neurons and no extraneous loss of neurons from cortex or hippocampus (Bilkei-Gorzo, 2014). This shows that neither model demonstrates all pathologies of AD, as such employing both to study the effects of leptin$_{116-130}$ may produce more robust data about its *in vivo* potential in AD.

During testing of healthy mice with leptin$_{116-130}$ we saw no side-effects on exploration, activity levels, health or weight of the animals. Longer-term exploration of potential side effects should also be undertaken. As leptin has been linked to both endometriosis (Alvarez *et al.*, 2014; Younger *et al.*, 2016) and breast cancer (García-Robles, Segura-Ortega and Fafutis-Morris, 2013; Chang *et al.*, 2015; Rodrigo *et al.*, 2017), it is important to understand if the leptin fragment can avoid these off-target actions. This could be studied by assessing pain sensitivity and tumour formation in healthy mice over longer time periods. Some previous studies have used long-term leptin treatments on mice. Whilst none of these studied the side effects, I have highlighted they did show leptin treatments can produce side effects long-term not seen in shorter experiments. For example, after 15 days of i.p. leptin injections meiotic index and spermatogonial nucleus diameter decreased in mice testicular tissue and further decreased after 30 days (Esmaili-Nejad, Babaei and Kheirandish, 2015); peripheral leptin treatment in C57BL/6J mice did not significantly increase plasma leptin levels until week 8 of treatment (Surwit *et al.*, 2000);

and in ob/ob mice leptin treatment initially decreased body mass but after 30 days this stabilised and additional treatment did not impact body fat (Eiden, Simon and Schmidt, 2005). Together these studies suggest long-term side effects should be investigated after 15-30days of daily leptin treatment. It is also important to note that in my memory study male mice were used whereas these side-effects are predominantly related to female health. Sex differences in memory have been suggested, with male rats more rapidly increasing response accuracy on an operant delayed spatial response alternation task (van Hest *et al.*, 1988) and in humans have better performance on visual-spatial tasks (Voyer, Voyer and Saint-Aubin, 2017) but females demonstrate better episodic memory (Spalek *et al.*, 2015) and female mice and rats demonstrate better performance on working memory tasks (Bimonte *et al.*, 2000). As such it is important to assess if there is a gender difference in the response to leptin$_{116\text{-}130}$ especially regarding side-effects. This future work would help to further elucidate the action of leptin fragments and their benefits to AD. These experiments with leptin and leptin$_{116\text{-}130}$ have laid important groundwork for an exciting potential therapeutic in AD. This thesis has demonstrated how a natural peptide hormone can mirror the beneficial effects of a previously promising treatment which has been disregarded due to side-effects. In addition, it has paved the way towards compounds which could realistically become oral therapeutics.

The second results chapter (6) aimed to identify neuroprotective, AD-modulative and cognitive enhancing effects of CCK1R agonism as this receptor has been linked to memory (Nomoto *et al.*, 1999; Xue-Liang Li *et al.*, 2002; Matsushita *et al.*, 2003) cell-survival signalling pathways (Zhou *et al.*, 2014) and pro-survival effects (Buscail *et al.*, 1995; Carrillo *et al.*, 2009; Lavine *et al.*, 2010). It was found 2nM A71623 consistently protected against cell death both in undifferentiated stressor conditions and fully differentiated cells under neurodegenerative conditions, seen to be signalled via STAT3. Studying A71623's impact on NS and OS revealed A71623 independently decreased NO levels in culture, an effect that was attenuated by inhibiting nNOS. Further A71623 decreased iNOS in protein extracts. As

such it appears A71623 could be decreasing NO in culture by decreasing iNOS but when an nNOS inhibitor is also applied this leads to a compensatory mechanism. A71623 was also shown to have potential as an AD therapeutic in its abilities to decrease tau phosphorylation at Ser262 and may impact ABAD levels. Whilst clear evidence of A71623's abilities to act as a cognitive enhancer were not elucidated, data suggests mice receiving this treatment performed better on the episodic memory task as they performed above chance levels whereas the controls did not. This data shows for the first time that activation of the CCK1R is protective against cell death and can reduce tau phosphorylation which is a major pathology in AD, as such this is a new and relevant area of research for therapeutic development.

Future work on A71623 should investigate its relationship with NO to understand its neuroprotective effects and roles within inflammation and learning and memory. One experiment that could be carried out on samples already gathered is to explore levels of NO and NOS in brain protein extracted from mice treated with A71623 as part of the OPC memory task. This would demonstrate how A71623 treatment directly affected NO production *in vivo*. This data could also be used to identify a correlation between NOS levels and performance on the memory task. Electrophysiology could be employed to record changes in LTP and LDP in hippocampal slices in response to A71623 treatment and NOS inhibitors. On-going electrophysiological experiments by collaborators with A71623 treatment has shown it to be a highly potent cognitive enhancer. This supports the trend in my data which shows better performance on the OPC task by A71623 treated mice compared to saline controls. NO has a role both in learning and memory and as an RNS in ageing. Understanding A71623's modulation of NO will aid understanding both of CCK1R's role in memory and in its neuroprotective abilities. Furthermore, NO has a role in inflammation, as such, CCK1R agonisms modulatory role may also come into play here. In order to investigate if CCK1R activation has a role in the immune response, A71623 could be applied to microglia cultures, and assessed via cell number and size, NO production and inflammatory cytokine levels (Savic *et al.*, 2014). As neuroinflammation is implicated in AD (Regen *et al.*, 2017) this may suggest even greater action of CCK1R agonism in AD treatment.

The OPC memory task suggests A71623 treated mice performed better than controls. Given ongoing work by collaborators supports this trend repeating the OPC task with more animals may produce a significant result. Due to discovering a birth defect in one animal and others being removed from the study for exploring less than five seconds in the 1-minute trials, numbers of animals used in the final analysis were reduced, an effect which impacted the control group to a greater extent. As I saw no significant impact of A71623 treatment on CCK1R expression in the brain after treatment, an improvement that could be made to this study is using a within subjects design such that all mice receive both saline and A71623 treatment and a comparison of performance can be made within mice. This would account for any idiosyncratic effects which may be masking the data. A final consideration is that when mice were tested on the OPC task with leptin treatments they only underwent the build-up tasks over two days rather than four. Whilst testing over four days gave a more consistent result and improved performance on the build-up tasks, this also extended the testing period for the mice by six days. In the leptin experiment even though exploration times were similar to the A71623 experiment, discrimination indexes were much higher and more frequently positive. This may indicate that the refinement to the experiment of additional testing in the NOR, OP, OC tasks led to a testing fatigue in the mice, such that by the time they underwent the OPC task they no longer found the experiment interesting. However, the trend that A71623 has positive cognitive effects, suggests other types of memory could also be explored such as spatial memory through the Morris Water Maze or mice could be treated during the component parts of the OPC task (NOR, OP, OC) as these rely on different areas of the medial temporal network (Barker *et al.*, 2007; Wilson, Langston, *et al.*, 2013; Yeung *et al.*, 2019) as such areas which feed into the hippocampus may also be involved in A71623's memory effects.

Similarly to leptin, testing A71623 on AD mouse models would indicate if it is able to alter AD pathology *in vivo* even if its effects on memory are unfounded. Whilst a treatment with potential cognitive enhancing effects would be most beneficial to patients, one that can halt disease progression and hence prevent any further memory loss is greatly needed. A71623 demonstrated it decreases tau phosphorylation under Aβ conditions, and as such demonstrates promise of disease-modifying effects.

A recent study demonstrated higher CCK levels in CSF correlated with both higher total tau and p-tau (Thr181) (Plagman *et al.*, 2019). This shows an *in vivo* relationship between CCK and tau and suggests other phosphorylation sites should be explored as well as downstream pathways which may play a role in tau phosphorylation e.g. glycogen synthase kinase-3 (Hanger *et al.*, 1992). Altogether this data shows that CCK1R is a strong drug target for an AD therapeutic. CCK2R's greater expression throughout the brain may suggest it would be a good drug target for neurodegenerative diseases. However, CCK2R is linked to hypolocomotion (Weiland, Voudouris and Kent, 2004) and increased anxiety (Li *et al.*, 2013) both of which would be greatly detrimental side-effects. Whereas in addition to my work demonstrating CCK1R agonisms protective effects it has also been linked to neuronal cell growth and proliferation (Langmesser *et al.*, 2007; Lassman *et al.*, 2010; Sui *et al.*, 2013) which suggests it can promote neuronal health.

The third results chapter (**7**) aimed to identify if co-treatment of A71623 with leptin or leptin$_{116\text{-}130}$ would prove a valid treatment strategy by investigating their combined neuroprotective properties and ability to modulate AD biomarkers. Further to this, other requirements for an AD treatment were considered in terms of receptor availability with age and effects of long-term treatments with our compounds. A synergistic relationship was identified but interestingly this was preferentially between A71623 and leptin$_{116\text{-}130}$ in neuroprotective effects and not A71623 with leptin. CCK and leptin have never been explored as synergistic protective treatments despite previous studies showing they can enhance each other's satiety effects. This data shows leptin's amino acids 116-130 are vital to its interaction with activated CCK1R and for the first time shows a differential action of leptin$_{116\text{-}130}$ to leptin. Further to this A71623 co-treatment with leptin$_{116\text{-}130}$ did not show additive activation of pSTAT3 this suggests co-treatment is activating other pathways to produce these effects. Whilst an alternative pathway was not elucidated within the scope of this thesis this result is exciting as it opens up the potential for co-treatments to have wider range of activity than A71623 or leptin$_{116\text{-}130}$ alone. Results

from the receptor expression experiments indicated effects of species, age, disease and brain region. This tells us that changes in receptors with both age and disease are highly regional and likely cell-type specific. This may suggest a co-treatment could be an even more effective therapy given leptin and CCK may have different capabilities to impact neuronal survival in different brain regions due to receptor expression changes. Finally, this chapter demonstrated in a pilot study the potential for long-term low-dose Aβ treatment of SHSY-5Y neurons to slowly induce cell death over time and with age. While this model requires some further optimisation, it demonstrated that all three compounds, especially A71623, have potential to be protective in culture long-term and that existing culture models can be adapted to be even more disease relevant. Overall, this chapter demonstrated the power of combination therapy and how it may be used to improve two treatments with great potential in the treatment of AD.

Moving forward, further investigation into co-treatments of both A71623 with leptin and with $leptin_{116\text{-}130}$ and why they produce differential effects may highlight new and interesting actions of these molecules which could be important in their uses as therapeutics. The differential action of co-treatment with leptin and $leptin_{116\text{-}130}$ may lend support to the fragment employing a different receptor or different ObR isoform. To further add to my work, co-treatments could be explored for their effectiveness against Aβ-induced toxicity and abilities to modulate AD biomarkers with an optimised protocol.

To further support the changes seen in receptor expression, protein can be extracted from the cat brain sections and ELISA's or western blots run to assess receptor protein quantity. Furthermore, it would be interesting to examine biomarker expression across age, CDS and species by looking at changes in the AD biomarkers across the cat brains but also in the aged SHSY-5Y and rat cortex samples. This could elucidate how these different populations change with age and if there are more similarities in animals which both naturally develop an Alzheimer's-like disease (humans and cats) than one which does not (rat). It may also be possible to correlate these changes in biomarkers with receptor changes

in specific brain regions of the cat to see if there is a potential relationship. This work suggests that leptin or CCK1R agonism could be treatments against CDS in cats. CCK1R expression especially saw very little change with age or CDS in cats, as such receptor availability suggests a treatment would have efficacy throughout the disease.

The question of sex differences can also be explored here. The SHSY-5Y cell cultures were originally taken from a female patient whereas the rat cortex samples were male. As such the difference in CCK1R expression may be due to sex rather than species. From the cat brains; two young, one old, and no CDS brains were male, all others were female, it would therefore be interesting to remove the males from the analysis and see how this effects the differences between young and old cats. Interestingly, sex differences in CCK concentrations in different brain regions have been seen in male and female rats, specifically in the ventromedial hypothalamic, medial and lateral preoptic, ventral tegmental, entorhinal and cortical areas (Frankfurt *et al.*, 1985). This is further influenced by the reproductive cycle and estradiol-17-β application, to dissected mediobasal hypothalamus from female rats, enhances CCK release (Micevych, Matt and Go, 1988). This demonstrates there is a direct influence of sex and related hormones on CCK in the brain. Whilst for leptin a less direct comparison between male and female brains has been made, work shows ObR gene expression is decreased in the arcuate nucleus with estradiol treatment and OVX increases expression (Bennett *et al.*, 1998), demonstrating leptin receptors in the brain can also be influenced by sex. Together this work shows the importance of sex. These differences are often overlooked in the development of new therapeutics, with male animal models being the industry standard and early drug trails often targeting male participants in order to avoid the influence of sex. As we know that males and females are affected by AD differently, with female risk increasing substantially post-menopause (Henderson and Brinton, 2010; Scheyer *et al.*, 2018), this shows how sex differences should be considered at all stages of the drug development process and that females should not be assumed to respond to treatments in the same manner as males.

The final experiment of this thesis explored the use of the ageing cell model and low-dose long-term Aβ treatment in order to establish a new model for testing AD therapeutics which may mimic the slow onset. Whilst this was a pilot study and as such did not reach significance, trends suggest this model was successful. To build on this, a study of ageing markers in the SHSY-5Y neurons across the time period used, such as OS by 8-hydroxy-guanosine detection (Wolf *et al.*, 2002), or investigation into the changing synaptic network using synaptic markers e.g. synapsin and PSD-95 (Micheva *et al.*, 2010; Broadhead *et al.*, 2016; Nery *et al.*, 2017), would allow insight into how these cells reflect ageing *in vivo*. As this model employs the same concentration of Aβ used to induce biomarkers in other experiments, optimisation of that protocol could be extended to include 2- and 3-week treatment periods which would demonstrate what happens in these cultures long-term. This model is in the early stages of development, but these preliminary results suggest this is an exciting new method which will allow even greater exploration of therapeutics on neurons long-term and reduce work in animal models. It will also allow us to more accurately explore use of treatments at different time points, for example, if there is a difference in effectiveness if cells are pre-treated or if treatment does not start until cells have been in Aβ-treated culture first. This will enable us to understand efficacy of treatments with diagnosis at different stages of AD.

Building on this, alternative methods of measuring viability could be employed. Throughout this thesis, whilst a cut-off criteria of 15% was used to define a successful reduction in viability (either 15% increase in LDH or decrease in CV and MTT assays), the variability and change in viability required to reach significance in any given experiment varied greatly. For example in **Figure 52** 10μM copper chloride treatment increased LDH release 37.3±6.292% relative to the control condition which was unable to produce a significant effect, however in **Figure 38** an increase in LDH of 22.5±5.86% by copper was significant. These assays can be impacted by culture conditions e.g. serum used in culture has LDH activity and MTT can be cytotoxic causing cell death during incubation (Aslantürk, 2017).This variation

leads to some difficulty in interpretation of the results, which is why, following the first experiment, at least two assays were used to confirm improvements in viability. However, using assays to measure viability in addition to colorimetric assays, such as; dye exclusion, fluorometric, or luminometric assays, may add clarity to results (Aslantürk, 2017). The live/dead cell assay is a widely used fluorometric assay which employs calcein-AM to detect live cells and propidium iodide to stain dead cells. Calcein is cleaved from calcein-AM by esterases in live cells emitting green fluorescence (Tawakoli *et al.*, 2013; Pfeffer and Fliesler, 2017), whereas propidium iodide is a nuclear stain which is unable to pass through intact membranes of live cells and fluoresces red (Tawakoli *et al.*, 2013; Stiefel *et al.*, 2015). Using this technique live and dead cells can be simultaneously detected in a single culture and can be performed on unfixed plates thus is unaffected by fixation or wash steps. Employing a live/dead cell assay to compliment the current viability assays would lend further clarification to the viability results.

To conclude, this thesis has demonstrated for the first time that a fragment of the leptin hormone can mirror the neuroprotective abilities of the full-length hormone and that both leptin and leptin$_{116\text{-}130}$ have cognitive enhancing abilities *in vivo*. This shows a natural leptin peptide has great potential as a therapeutic in AD because it can mimic the beneficial effects of leptin and may be specific in its action. In addition, I have shown that agonism of the CCK1R is also neuroprotective, modulates NS and p-tau, and trends suggests it enhances cognition. CCK1R has been previously unexplored for its potential in neurodegeneration due to its lesser expression in the brain compared to CCK2R but this work proves its beneficial effects and reveals an exciting avenue for future therapeutics. This data demonstrates that as yet unexplored hormones and their receptors can pave the way for new treatments in AD. Together, these chapters show that by considering the endocrine theory of ageing and identifying neurodegenerative diseases, such as AD, as dysfunctions occurring at a system rather than cellular level, new treatments can be uncovered which are effective at combating pathology. This

work provides a new insight into tackling AD so that treatments developed could succeed where others have failed.

Building on this, the final chapter provides a new strategy for treatments by studying naturally occurring synergy in hormonal systems. I have demonstrated that CCK1R agonism can work with leptin$_{116\text{-}130}$ to reduce cell death and induce mitosis. Furthermore, this was achieved at substantially lower concentrations than protection was previously induced with individual treatments. This adds to the benefit of these treatments as it increases the likelihood of tolerance and decreases the potential for side-effects. As expression of the receptors for these treatments are differently effected across brain regions age and onset of an AD-like disease, this shows how a co-treatment could also have great effect across the brain as areas which have lower expression in one receptor may not change in expression of the other, as such the treatment would still have an impact. Therefore, leptin$_{116\text{-}130}$ and CCK1R agonism should be further explored for their potentials as AD therapeutics by use of both cellular and animal-based models, and moreover, a focus should be placed on the benefits of co-treatment as a strategy which can provide greater efficacy and reduced side effects. As such this thesis has shown that hormones have potential use as therapeutics in AD and therefore greater investigation into endocrine system alterations with age and disease may give greater insight into preventative measures.

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9.1 CLINICAL TRIALS:

Biomarker Qualification for Risk of Mild Cognitive Impairment (MCI) Due to Alzheimer's Disease (AD) and Safety and Efficacy Evaluation of Pioglitazone in Delaying Its Onset (TOMMORROW). (2018). Retrieved from: https://www.clinicaltrials.gov/ct2/show/NCT01931566?term=pioglitazone%2C+alzheimer&rank=1 Identification No. NCT01931566

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