MONASH University

Characterisation of the paradigms used to assess IRAP inhibitors as novel cognitive enhancing agents

Benjamin John Seyer

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Abstract

As the prevalence of dementia is rising, there is a pressing need for novel cognitive enhancing agents (nootropics). One possibility is targeting Insulin-Regulated Aminopeptidase (IRAP), with inhibitors of this enzyme documented to enhance cognition, however, their mechanism remains unknown. This work sought to characterise the paradigms used to assess the IRAP-Is, in order to provide clues as to the potential mechanisms governing this cognitive enhancement.

To study their effects on memory, the most common delivery method of the IRAP-Is is through chronic intracerebroventricular cannulas. Whether the surgery procedure itself could be inducing memory deficits is unknown and potentially confounds conclusions drawn regarding the action of IRAP-Is. This work demonstrated that following surgery there was no impairment to either spatial working or recognition memory (two common tasks used in the testing of the IRAP-Is). Inflammatory mediators have been known to impair memory, however, immunohistochemistry showed that there was minimal immune cell activation and spread from the cannulation site and this was confirmed by real-time PCR. These results indicate that IRAP-Is are likely enhancing normal memory, rather than rescuing a deficit, suggesting they are true nootropics.

Due to the biphasic dose response of the IRAP-Is, combined with the time consuming nature of behavioural testing, screening for novel IRAP-Is with cognitive enhancing properties is difficult due to the biphasic dose response curve, thus making determination of dose difficult. I sought to develop a rapid *in vitro* bioassay that could screen for novel IRAP-Is while also investigating the underlying mechanism of action. Treatment of primary hippocampal neuron cultures with IRAP-Is resulted in an increase in dendritic spine density (small neuronal outgrowths responsible for communication and correlated directly to cognitive status). Using radiolabelled deoxy-glucose, IRAP-Is induced glucose uptake with the same biphasic dose response as spine growth and both of these actions were abolished by blockade of the inducible glucose transporter, GLUT4. This provided not only a mechanism by which the IRAP-Is were enhancing

cognition, but also that measurement of glucose uptake in hippocampal cultures serves as a rapid *in vitro* bioassay.

The final chapter investigated the potential of IRAP-I treatment in Alzheimer's Disease (AD) using the transgenic 5xFAD mouse model. While reductions in cerebral glucose uptake and dendritic spines are key features of human AD and possible pathways by which IRAP-Is could have therapeutic action, the phenotype of the AD mice with regards to cerebral glucose uptake is unclear. Using autoradiography to measure radiolabelled deoxy-glucose uptake, the 5xFAD mouse cerebral metabolic phenotype did not match the human condition. However, golgi-cox staining to assess dendritic spines revealed that the 5xFAD mouse displayed a reduction in spine density that was rescued by chronic peripheral IRAP-I treatment. Further, this treatment also enhanced density in wild-type animals.

In summary, IRAP-Is are able to enhance memory and spine density in unchallenged states, demonstrating their potential as true nootropic agents and the development of the *in vitro* bioassay will allow rapid screening of novel compounds. The ability of IRAP-Is to enhance memory, neuronal glucose uptake and dendritic spines places them ideally as a therapeutic in AD, a disease that features deficits in each of these areas.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature

Print Name: BENJAMIN JOHN SEYER

Date: 31/10/2017

Publications during enrolment

Seyer, B., et al. (2016). "Cannula implantation into the lateral ventricle does not adversely affect recognition or spatial working memory." Neuroscience Letters **628**: 171-178.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer-reviewed journal. Whilst the work with the chapter is the same, much of the discussion is relevant only to thesis, thus the published article is attached in the appendix. The core theme of the thesis is an investigation of the paradigms surrounding IRAP inhibitor testing. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Physiology, Monash under the supervision of Siew Yeen Chai.

In the case of chapter 2, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-authors, Monash student
2	*Characterization of the behavioural consequences of intra-cranial-ventricular cannulation*	*Published*	*80%. Concept, collecting data, writing first draft and finalising*	*1) Siew Chai, input into manuscript 10%* *2) Vi Pham, Performed RT-PCR 5%* *3) Anthony Albiston, manuscript proof reading, 5%*	*No* *No* *No*
3	*IRAP inhibitors mediate increases in dendritic spine density by enhancing glucose uptake through GLUT4*	*Unpublished*	*85%. Concept, collecting data, writing first draft and finalising*	*1) Siew Chai, Input into manuscript, 10%* *2) Shanti Diwakarla, determination of functional spine proportion, 5%*	*No* *No*

4	*Evaluation of the 5xFAD Alzheimer's mouse as a suitable model to assess the therapeutic potential of the IRAP inhibitors*	*Unpublished*	*80%. Concept, collecting data, writing first draft and finalising*	*1) Siew Chai Input into manuscript, 10%* *2) Peta Burns, surgery and behavioural testing of 5xFAD, 10%*	*No* *No*

Student signature:
Date: 31-10-17

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work.

Main Supervisor signature:

Date: 31-10-17

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To the IRAP lab, Leelee and Pikying, I am very grateful for your time and expertise and know I would not have gotten to where I am without your help. In particular, many thanks to Peta, Vi and Shanti, for teaching me many of the techniques that made this project possible, and most of all for your patience when dealing with my (many) stupid mistakes.

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Abbreviations

ARB	Angiotensin Receptor Blocker
ACE-I	Angiotensin Converting Enzyme Inhibitor
AD	Alzheimer's Disease
AngIV	Angiotensin IV
ANOVA	Analysis of Variance
Aβ	Amyloid Beta
CA	Cornu Ammonis
CGU	Cerebral Glucose Uptake
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DIV	Days *In Vitro*
FDG	Flurodeoxyglucose
i.c.v	Intracerebroventricular
IRAP	Insulin-Regulated Aminopeptidase
IRAP-I	Insulin-Regulated Aminopeptidase Inhibitor
KO	Knock Out
LTP	Long-term potentiation
MMP	Matrix MetalloProteinase
mTOR	Mammalian target or rapamycin
NOR	Novel Object Recognition
PBS	Phosphate Buffered Solution
PET	Positron Emission Tomography
PFA	Paraformaldehyde
RPM	Revolutions Per Minute
SAT	Spontaneous Alternation Task
SEM	Standard Error of the Mean

Chapter 1

Literature Review: IRAP, memory and Alzheimer's disease

1.1 Introduction

With an aging population one of the greatest challenges facing society is how to treat age-related diseases one of the most prominent being dementia. Interestingly, with improved primary healthcare and livelihood, the incidence of dementia has remained stable or even declined in some European countries (Wu et al. 2017). Despite this, the disease still incurs a great personal cost to both individual and family. Further, there are substantial monetary costs to the community associated with the disease. Worldwide, based on a population of 46.8 million affected, the cost of dementia was estimated to be US$818 billion in 2015, an increase of 35.5% already from 2010 (Prince 2015).

Alzheimer's disease (AD) is the most common form of dementia, accounting for up to 70% of all cases (Wimo et al. 2010). While several lifestyle factors such as obesity and smoking have been associated with an increased risk for the disease (Prince 2015), there is no definitive cause and the need for an effective drug therapy is pressing. Following decades of research, many drugs have displayed promising results in preclinical trials, often showing substantial reductions in pathology, however these have all invariably gone on to fail in clinical trials (Green et al. 2009, Quinn et al. 2010, Hung et al, 2017).

A promising target has emerged in the protein Insulin-regulated aminopeptidase (IRAP), with inhibitors of the protein shown to enhance memory and learning in several behavioral tasks. This review will discuss the discovery of IRAP, initially identified as the binding site of the endogenous hormone Angiotensin IV, the synthesis of novel inhibitors, before delving into the potential mechanisms that may underlie the cognitive enhancing effects of the inhibitors as well as the challenges that currently face the area.

1.2 Overview of the Insulin-regulated aminopeptidase, IRAP

IRAP is highly expressed in several regions of the brain crucial for cognitive function, particularly the hippocampus and basal forebrain (Chai et al. 2000, Fernando et al. 2005). Further, inhibitors of IRAP have been shown to enhance cognition in behavioral memory tasks (Albiston et al. 2008), however underlying mechanism of this enhancement remains unclear. It is necessary to first understand the structure, distribution and suggested physiological

functions of IRAP, in order to then predict the mechanism that may play a role in cognitive enhancement.

1.2.1 The structure of IRAP

Consisting of 1025 amino acids with three domains, IRAP is a Type II transmembrane aminopeptidase (Lausten et al. 1997). The hydrophilic N-terminal intracellular domain consists of 110 amino acids and contains a number of motifs involved in trafficking, such as two dileucine motifs followed by acidic clusters (Keller et al. 1995). This cytosolic tail of the protein is responsible for much of the endosomal localization of IRAP with short chimeric forms of the enzyme displaying the same localization as the full-length version (Subtil et al. 2000, Hou et al. 2006). The N-terminal is also seen to interact with a number of tethering proteins within the cell (discussed below). This domain is followed by the transmembrane component of 22 residues and then the extracellular domain consisting of 893 residues which contains the catalytic site with both a GAMEN substrate recognition sequence and a $HEXXH(X)_{18}$ E Zn^{2+}-binding motif (Keller et al. 1995). IRAP is also a glycoprotein as evidenced by the reduction in size following treatment with PNGase F, an enzyme that removes N-linked carbohydrates (Bernier et al. 1998, Zhang et al. 1999),, and stepwise reduction demonstrates a least 2 linkages. Interestingly, the size of IRAP located in the hippocampus is smaller than other regions (150kDa Vs 165kDa, due to glycosylation) including the bladder, thymus, kidney, aorta and adrenal glands (Zhang et al. 1999), however the deglycosylated protein size is the same across all regions.

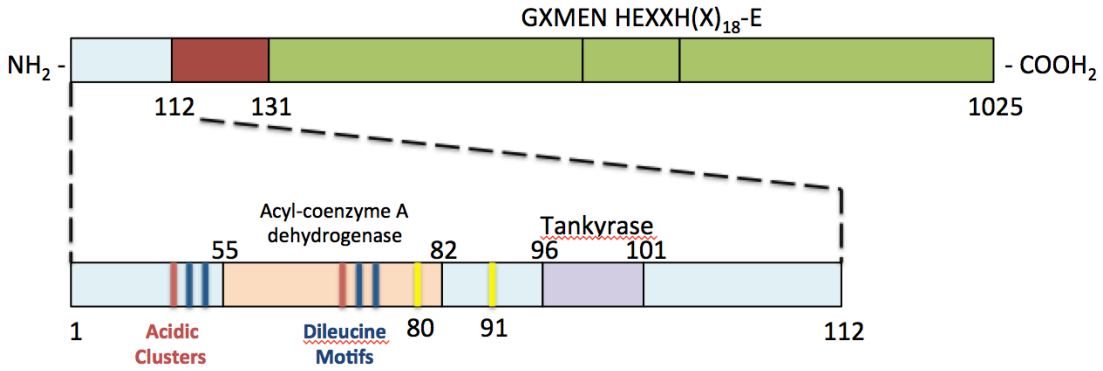


Figure 1-1 Schematic of insulin regulated aminopeptidase

The extracellular catalytic domain (green, amino acids 131 - 1025) contains the Zn^{2+}-binding [HEXXH(X)$_{18}$-E] and exopeptidase (GXMEN) motifs. The intracellular domain (light blue, amino acids 1 – 112) contains the dileucine motifs (blue bars) and the acidic clusters (red bars) as well as the acyl-coenzyme A dehydrogenase (amino acids 96 – 101), protein kinase C-ζ (amino acids 80 and 91, yellow bars) and tankyrase (amino acids 96 – 101) recognition and binding sites.

Adapted from Albiston et al. 2003

These various domains combine to give IRAP a diverse range of endogenous functions, a fact that is exemplified in its initial discovery by three separate groups. First as a marker of insulin responsive vesicles containing the glucose transporter $GLUT_4$ where it was designated vp165 (Keller et al. 1995). It was then postulated to be a degradative enzyme for oxytocin, leading to it also being referred to as oxytocinase (Rogi et al. 1996) before it was finally it was identified as the binding site for angiotensin IV (Albiston et al. 2001).

1.2.2 Distribution

Similar to its diverse structure, IRAP's distribution is also quite broad, with expression seen in many organs throughout the body including the;

- Heart (Hanesworth et al. 1993, Harding et al. 1994),
- Kidney (Muse et al. 2007, Albiston et al. 2011),
- Adipose tissue (Waters et al. 1997),
- Placenta (Nomura et al. 2005) as well as umbilical tissue (Nakamura et al. 2004)
- Skeletal muscle (Garvey et al. 1998),

- Spleen, particularly within immune cells such as mast cells (Liao et al. 2006) as well as B cells, T cells and dendritic cells (Saveanu et al. 2009),
- as well as vascular tissue (Harding et al. 1994)

Most importantly for the work presented in this thesis is the distribution of IRAP within the brain. IRAP's expression correlates well with its role in learning and memory as it has concentrated expression in several key areas such the cornu ammonis (CA regions) of the hippocampus, basolateral amygdala and frontal, prefrontal, insular and entorhinal cortices (Fernando et al. 2005). This is a trend replicated across multiple species (Miller-Wing et al. 1993, Moeller et al. 1996, Burns et al. 2004) including humans (Chai et al. 2000). Of note is that in all of the above cases, IRAP appears to reside in endosomal vesicles in the basal state where it recycles slowly but can translocate to the cell membrane upon stimulation.

1.2.3 Multiple roles of IRAP

1.2.3.1 Identification of IRAP as the binding site for AngIV

Most relevant to its role in enhancing memory was the discovery that IRAP is the binding site for angiotensinIV (AngIV), a hormone seen to produce pro-cognitive effects when injected into the ventricles of the brain Wright et al. 1999). Radioligand binding has demonstrated that AngIV binds with high affinity to IRAP (Lew et al. 2003, Demaegdt et al. 2009) and that the pattern of IRAP expression and the binding profile of AngIV in the brain are closely aligned with one another (Albiston et al. 2001). Further, heterozygous IRAP knockout mice display reduced binding for AngIV as determined by *in vitro* autoradiography using 125 I-Nle AngIV binding while homozygotes show a complete loss of binding (Albiston et al. 2010) as seen in Figure 1-2.

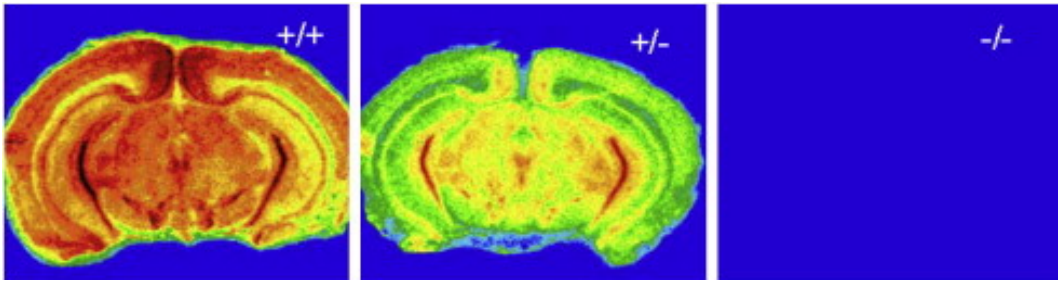


Figure 1-2 Binding of AngIV in the IRAP KO mouse brain demonstrated via autoradiography

These images demonstrate the progressive loss of AngIV binding in the brains of; wild-type (+/+), heterozygote (+/-) and homozygote (-/-) IRAP knock-out mice (Albiston et al. 2001). Images produce via 125 I-Nle AngIV autoradiography

It should also be noted here that while IRAP is often referred to as the AngIV receptor within the literature, AngIV is actually binding to the catalytic domain of IRAP (Herbst et al. 1997) and enzyme kinetic analysis has demonstrated that AngIV is competitive inhibitor of IRAP (Albiston et al. 2001). There has been debate within the literature as to whether IRAP is the specific binding site of AngIV, with some suggestion that AngIV may bind to c-MET (Wright et al. 2008). However evidence for this is circumstantial at best and only evidence presented within review articles.

1.2.3.2 Oxytocinase

IRAP is expressed within the placenta where it is known as oxytocinase, or, placental leucine aminopeptidase. Here IRAP is cleaved at the recognition sequence F_{154}/A_{155} (Iwase et al. 2001, Ito et al. 2004) and a circulating form of the enzyme released during pregnancy, reaching maximum levels near term (Gazarek et al. 1975, Mizutani et al. 1992). Indeed, IRAP immunotitration is also able to reduce the peptide degrading activity of human pregnancy sera (Mizutani et al. 1995), indicating that the enzyme may play a role in regulating peptide levels throughout pregnancy. Circulating IRAP levels during pregnancy have also been suggested to be a marker of fetal health (Tian et al. 2016).

Despite this, in IRAP KO mice, there are no differences observed in gestation period or pup number, however, circulating IRAP is not detected even

in wild-type pregnant mice (Pham et al. 2009). The authors note that in order for IRAP to be released into circulation it must be cleaved at Phe^{154} Ala^{155} (Iwase et al. 2001), however this sequence is not conserved across species (Rosenbloom et al. 1975). Thus while the proteins may be *largely* homologous across species, the role within pregnancy may be varied.

1.2.3.3 Intracellular Signaling

While IRAP possesses a cytosolic tail with motifs expected to be involved in signaling and trafficking, there is conflicting evidence as to the effect of the inhibitors on a variety of second messenger systems. In human HK-2 cells (Handa 2001) and bovine kidney cells (Handa et al. 1999, Handa 2000), treatment with AngIV induces an increase in intracellular calcium levels. These results are contrasted by studies demonstrating that AngIV treatment of chick cardiomyocytes (Baker et al. 1990), human collecting duct cells (Czekalski et al. 1996) and porcine proximal myocytes (Chen et al. 2001) does not affect intracellular Ca^{2+} levels or cAMP and CGMP.

1.2.3.4 Antigen cross-presentation

IRAP is also suggested to play a role in antigen cross presentation after its observed localization to the endosomal compartments of dendritic cells (Saveanu et al. 2009). Cross-presentation involves the uptake, processing and presentation of extracellular major histocompatibility complex class 1 molecules to $CD8^+$ cells (Bevan 2006). IRAP is not only present in the dendritic cells responsible for this action, but is also essential to their function, with IRAP deficiency resulting in compromised cross presentation (Weimershaus et al. 2012). IRAP shares motifs and high sequence homology with other antigen trimming proteins such as endoplasmic reticulum aminopeptidases 1 and 2, both of which are also part of the M1 aminopeptidase family (Tsujimoto et al. 2005), however IRAPs substrate specificity appears to be broader (Georgiadou et al. 2010). It is also distinct from these enzymes by the presence of its N-terminal cytoplasmic domain.

1.2.3.5 Pathophysiological expression of IRAP

Following on from this expression in dendritic cells, IRAP also appears to play a role in numerous pathological states. In cancer, the density and/or activity of IRAP correlates with cancer malignancy (Shibata et al. 2005), stage and grade (Larrinaga et al. 2007) as well as invasiveness (Cohen et al. 2008). In the vasculature, IRAP expression is increased within atherosclerotic lesions following a high fat diet (Vinh et al. 2008), while the global IRAP$^{-/-}$ mouse has improved outcomes following thrombos induction (Numaguchi et al. 2009) and ischemic stroke (Pham et al. 2012).

Of particular interest to the current work is the potential role IRAP plays in the neuroinflammatory response. In our laboratory, strong ^{125}I-Ang IV binding has been noted in the white matter of the sheep spinal cord following compression injury, appearing to be associated with activated astrocytes (Moeller et al. 1996). One week following stab injury to the cerebral cortex, IRAP expression also appears within activated astrocytes (*Fernando,* unpublished) and also in the days following induction of ischemic stroke (*Telianidis,* unpublished).

Overall, in the periphery there is good evidence to support the role of IRAP in mediating pathological effects following a variety of insults. While the evidence is more tenuous within the brain, it does remain a factor to consider when investigating the possible mechanisms of IRAP-I mediated cognitive enhancement, as will be discussed in detail in chapter 2.

1.2.3.6 Interaction with Matrix Metalloproteinases

IRAP also appears to interact with matrix metalloproteinases (MMPs) in a number of different paradigms. The MMPs are a group of extracellular zinc-dependent enzymes that are widely distributed throughout the body and play a major role in matrix remodeling.

IRAP inhibitors (including AngIV) reduced the infiltration and proliferation of trophoblasts in the first trimester of pregnancy while also reducing the activity of MMP-9 (Cohen et al. 2008). Meanwhile, IRAP deletion in mice resulted in decreased expression of MMP-2 and increased plaque accumulation in the aorta (Numaguchi et al. 2010). Of note, while the plaque

build up in the IRAP KO animals may suggest a possible negative side effect of IRAP-I treatment, the opposite has been observed, with treatment with AngIV reducing plaque accumulation (Vinh et al. 2008). This difference is likely due to developmental deletion of IRAP having negative consequences while inhibition in the adult animal is beneficial.

Correspondence from our collaborators has also indicated that in cardiac fibroblasts, IRAP-I treatment is able to increase the expression of MMP-2, while in an angiotensin-II induced model of hypertension, both IRAP deletion and inhibitor treatment increased the expression of MMP-13 (*Lee,* unpublished*)*. These interactions are of particular interest here as MMPs also play a crucial role in the formation of memory, as will be discussed below.

1.2.3.7 GLUT4 Vesicle Trafficking

As discussed earlier, as well as being termed oxytocinase, IRAP was also initially designated as vp165 when it was identified as a protein seen to traffic with GLUT4 in adipocytes (Keller et al. 1995) and is indeed the only protein to accompany GLUT4 throughout its trafficking cycle. IRAP appears to be responsible for proper vesicle trafficking and is seen to interact with a number of proteins involved in vesicle tethering through its intracellular N-terminal domain. GLUT4 vesicles appear to be bound at an intracellular site before recruitment to the plasma membrane upon insulin stimulation. As such, these vesicles must interact with the cytoskeleton of the cell, a fact that has been demonstrated by several studies showing that insulin stimulation is sufficient to cause reorganization of actin filaments (Omata et al. 2000, Kanzaki et al. 2001, Tong et al. 2001). In line with this, IRAP has been demonstrated to bind to FHOS (Forming homologue overexpressed in spleen) (Tojo et al. 2003) which in turn affects the cytoskeleton via its interaction with profillin IIA. Overexpression of FHOS within L6 cells was able to cause a 2-fold increase in basal glucose, likely by disrupting the IRAP-FHOS binding that tethers GLUT4 vesicles within the cell (Tojo et al. 2003).

IRAP has been shown to bind to both tankyrase 1 (Sbodio et al. 2002) and 2 (Chi et al. 2000), poly-ADP ribose polymerases that have previously been associated with telomere function, however, are now known to play a role in

GLUT4 vesicle trafficking. Upon stimulation tankyrase translocates to the plasma membrane, similar to GLUT4 and IRAP, suggesting it may play a role in targeting the proteins to the correct endosome. This is supported by evidence that tankyrase knockdown results in reduced translocation of both GLUT4 and IRAP to the plasma membrane in response to insulin stimulation and alters the basal distribution from lighter to heavier endosomal fractions (Yeh et al. 2007). Yet another vesicle tethering protein found to interact with IRAP is p115, a golgin protein family member (Hosaka et al. 2005). Both p115 and GLUT4 are heavily co-localised in the perinuclear region of cells, although only GLUT4 translocates to the cell surface upon insulin stimulation. Over-expression of the p115 N-terminus in 3T3-L1 adipocytes abolishes GLUT4 redistribution however. It is likely that this over-expressed form competes with endogenous p115 for IRAP binding and shifts the protein away from the normal insulin responsive compartments, thus disrupting GLUT4 trafficking.

1.2.3.8 Phenotype of the IRAP KO mouse

Particularly interesting in the context of the current work is the effect of IRAP deletion on memory and learning. Surprisingly, while IRAP inhibition by compounds results in an enhancement of memory (discussed below), deletion of the IRAP gene results in accelerated decline in spatial memory (Albiston et al. 2010), although recognition memory is spared. This decline in spatial memory could be due to altered brain development, indeed IRAP shows high expression in the subventricular zone during embryonic mouse development (Chai et al. 2001) and knockout animals appear to have reduced cortical thickness and impaired dentate gyrus formation (*Diwakarl*a, unpublished*)*. Despite this, the knockout mice are similar to their wild-type counterparts in terms of general health and spontaneous behavior.

In order to investigate this further, a forebrain specific inactivation of IRAP in post-mitotic neurons (thus, no interference with development) was investigated. These animals displayed a reduction in locomotor activity as well as impaired balance and coordination (Yeatman et al. 2016). While these animals demonstrated similar results to the global IRAP knockout mice in terms of impaired spatial reference memory, they also demonstrated impairment in

the novel objection recognition task, in contrast to the previous work. Of interest is that these animals maintained intact spatial working memory even in presence of impaired spatial reference memory (Yeatman et al. 2016). Thus, even the loss of IRAP in early post-natal life likely still has a detrimental effect on brain development, although the precise reason for the differences seen from the global IRAP knockout mice remains unclear.

1.2.3.9 Neuropeptide regulation

IRAP is able to remove the N-terminal amino acid residues from a wide variety of neuroactive peptides (Matsumoto et al. 2001), including vasopressin and oxytocin (Matsumoto et al. 2000), lys-bradykinin, met-enkephalin, dynorphin A (1-8), neurokinin A, neuromedin B, somatostatin and CCK-8 (Herbst et al. 1997, Matsumoto et al. 2000, Lew et al. 2003). It is notable that for IRAP to be able to cleave substrates it must move to the cell surface, as evidenced by stimulation of adipocytes with insulin (known to cause IRAP translocation to the cell surface) resulting in faster cleavage times for vasopressin (Herbst et al. 1997).

As yet, only vasopressin has some evidence to support its cleavage by IRAP *in vivo.* Endogenous levels of vasopressin were twice as high in IRAP knockout mice compared to their wild-type counterparts and clearance of peripherally injected vasopressin was also slower in the knockout animals (Wallis et al. 2007). Mirroring the *in vitro* work by Herbst and colleagues, stimulation with insulin increased the rate of clearance (Wallis et al. 2007). In humans, patients undergoing septic shock had a higher 28-day mortality when they expressed a single nucleotide polymorphism located in the regulatory region of the IRAP gene and this was accompanied by increased vasopressin clearance (Nakada et al. 2011).

1.3 The interaction of IRAP and memory

In addition to all of the roles discussed above, the reason that this work has chosen to focus on IRAP is due to its ability to enhance cognition in a variety of rodent memory tasks.

1.3.1 Memory enhancement by AngIV

Although it had not yet been identified as the binding site for AngIV, the first accounts of IRAP mediated memory enhancement were reported in the passive avoidance paradigm (Braszko et al. 1988, Braszko 2004, Braszko 2006, Braszko et al. 2008) where administration of AngIV into the cerebral lateral ventricles increased the time taken for rats to re-enter a dark chamber that was associated with an aversive foot shock and a similar effect noted in the conditioned avoidance task (Braszko et al. 1991, Wright et al. 1993, Braszko 2004, Braszko 2006). In addition to these emotive based paradigms, enhanced performance has also been noted in object recognition (Braszko 2004, Braszko 2006, Braszko et al. 2008) as well as on spatial memory tasks such as the radial arm maze (Braszko et al. 2008), Morris water maze (Wright et al. 1999) and Barnes circular maze (Lee et al. 2004). Treatment with AngIV is also able correct deficits that are induced by chronic alcohol exposure (Wisniewski et al. 1993), global ischemia (Wright et al. 1996), bilateral perforant pathway lesions (Wright et al. 1999) and disruptions to the septo-hippocampal cholinergic pathway (Pederson et al. 2001, Lee et al. 2004, Olson et al. 2004, Olson et al. 2010).

Lending further support to its ability to enhance cognition, one of the key cellular correlates of memory, long-term potentiation (LTP), is also positively influenced by AngIV, with increases seen in both the dentate gyrus *in vivo* (Wayner et al. 2001) and CA1 region *in vitro* (Kramár et al. 2001). This increase in LTP in response to AngIV appears to be mediated by N-methyl-D-aspartate channels and subsequent calcium entry (Davis et al. 2006). Further, blockade of voltage gated calcium channels abolishes the pro-cognitive effects of AngIV treatment in both the passive avoidance and novel object recognition tasks (Braszko 2017).

While the aforementioned studies have involved delivery of AngIV directly into the ventricles, there is also evidence to suggest that subcutaneous administration is able to enhance cognition in the novel object recognition task (Golding et al. 2010, Gard et al. 2012). These results are particularly interesting given that as a large peptide it would be predicted that AngIV is unable to cross the blood-brain barrier. Indeed, generation of novel compounds that are able to cross the barrier while retaining their ability to inhibit IRAP has been the subject of research (Wright et al. 2010) and these results warrant further investigation.

1.3.1.1 Possible role of the oxytocin in mediating AngIV effects

An accumulation of neuroactive peptides has been one of the proposed mechanisms underlying the enhanced cognition in response to AngIV treatment. As AngIV acts as an inhibitor of the catalytic activity of IRAP, this could allow substrates such as oxytocin to accumulate and potentiate memory. Rats treated with subcutaneous AngIV and oxytocin both exhibited enhanced memory during the novel object recognition task, an effect that was abolished with the co-treatment of an oxytocin antagonist (Gard et al. 2012). However, this antagonist was also able to block the cognitive enhancing effects of physostigmine, a cholinesterase inhibitor, suggesting that the antagonist was detrimental to memory in its own right, rather than specifically interfering with the action of AngIV. It is difficult to definitively conclude this however as both saline treated (control) animals and oxytocin antagonist animals performed at chance levels (i.e. further reduction in performance compared to the control animals was not possible). There has also been the suggestion that IRAP inhibition by LVV-H7 (another peptide inhibitor of IRAP) is able to produce anxiolytic effects by preventing the breakdown of oxytocin (da Cruz et al. 2017). Again, this was determined by co-administration of an oxytocin antagonist, however this would presumably abolish endogenous oxytocin activity regardless, further it is also notable that many studies do not report an anxiolytic effect with IRAP-I treatment.

1.3.1.2 Possible involvement of dopaminergic signaling

There also appears to be a contribution of the dopaminergic system toward the pro-cognitive effects of AngIV. Early evidence demonstrated that

treatment with AngIV increased motor stereotypies (Braszko et al. 1976) as well as motor activity in rats (Braszko et al. 1988) an effect known to be mediated by dopamine signaling. Indeed the cognitive enhancing effects of AngIV treatment were abolished by blockade of D_1 (Braszko 2004), D_2 (Braszko 2006), D_3 (Braszko et al. 2008) and D_4 (Braszko 2009) receptors. Further support for this notion comes from the relative pattern of expression of the dopamine receptor sub-types, high expression of D_1 and D_2 in the hippocampus and striatum correlating with impaired AngIV efficacy on spatial memory tasks in the presence of the associated dopamine receptor antagonists (Braszko 2004, Braszko 2006) but not in the presence of the D_3 and D_4 antagonists (Braszko et al. 2008, Braszko 2009), whose receptors show low expression in these areas (Mansour et al. 1995).

1.3.1.3 Conflicting reports of IRAP involvement in AngIV mediated memory enhancement

Some studies have suggested that the beneficial effects of AngIV administration are not due to its inhibition of IRAP activity. There are reported strain differences in the behavioral response to peripheral AngIV administration, whereby C57/Bl6 mice showed enhanced performance on the novel object recognition task while two others, the CD and DBA_2 strains did not (Golding et al. 2010). As AngIV inhibited the aminopeptidase activity of IRAP roughly equally across the strains it suggests that another factor was causing the enhanced cognition. However, this only precludes IRAP as the mediating factor if the cognitive enhancing effects are dependent on this catalytic activity (due to neuroactive substrate accumulation for example). It is possible that there also strain differences in the effect of AngIV on glucose uptake which has also been proposed as a mechanism of cognitive enhancement, however this was not assessed in the study.

1.3.2 Synthetic inhibitors of IRAP

Unfortunately, despite the promising results seen with AngIV, it remains unviable as a potential drug candidate. The peptide displays a certain level of binding promiscuity that not only makes it difficult to definitively claim IRAP as

the target protein behind the observed effects, but also increases the risks of harmful side effects.

Alternate binding sites observed for AngIV have been the enzymes; Angiotensin Converting Enzyme 1 (Lantz et al. 1991, Fruitier-Arnaudin et al. 2002) and Aminopeptidases A (Goto et al. 2006) and N (Garreau et al. 1998). In each of these cases it is able to inhibit the activity of these enzymes and it is possible that this may contribute to the bell shaped dose response observed to AngIV (and IRAP-I treatment more broadly) that will be discussed later. AngIV has also been shown to bind the AT_1 receptor at high concentrations (Lochard et al. 2004). Further issues arising from the use of peptides is their rapid degradation with AngIV showing a 68% reduction after a 20 minute incubation with rat mesangial cells (Chansel et al. 1998). In order to combat these issues, several synthetic non-peptide inhibitors of IRAP have been generated and shall be discussed below.

1.3.2.1 Synthesis of novel non-peptide IRAP inhibitors

The peptidomimetics are structural analogues of AngIV and are generated by our colleagues in Sweden. They are made via the addition of a macrocyclic ring at the N-terminal, displacement of several C-terminal residues by phenylaetic acid and the replacement of several amino acids by those with olefinic side chains (Andersson et al. 2010, Andersson et al. 2011). This leads to compounds that are less peptidic in nature compared to AngIV, which in theory increases selectivity for IRAP and reduces degradation. However these molecules are still of a very large size, some with a molecular weight >700 Daltons (Axén et al. 2006) and this may lead to issues in crossing the blood brain barrier when administered peripherally. These inhibitors also currently have no *in vivo* evidence to support their use as cognitive enhancers.

1.3.2.2 Inhibition of IRAP is able to enhance memory

While modification of the peptide inhibitors has shown some success, our lab has instead sought a different route. In silico screening against using a homology model of the catalytic site of IRAP several molecules were identified as potential inhibitors, the most promising to date being HFI-419 which has been demonstrated to have over 1000-fold selectivity for IRAP compared to

enzymes with a similar structure and has a reported K_i of 480nM (Albiston et al. 2008). Importantly, the previous memory enhancing results obtained with AngIV have been expanded upon with HFI-419, which is also able to enhance memory in both the spontaneous alternation and the novel object recognition tasks (Albiston et al. 2008). Despite these promising results, the specific mechanism by which the IRAP inhibitors exert their beneficial effects on cognition is still unknown and has been the subject of much research of late. Further, the pharmacokinetics of the current inhibitors are not ideal, in rats, brain to plasma ratio 5 minutes following tail vein injection of 5mg/kg HFI-419 was 0.39 while it could not be detected in the brain 60 minutes post administration

Another factor hampering research is the tight bell shaped dose response that the inhibitors display that makes *in vivo* screening for novel IRAP inhibitors that display pro-cognitive effects difficult.

To address these issues an *in vitro* assay is needed for high throughput screening. At a cellular level, IRAP inhibition enhanced the growth of dendritic spines (Diwakarla et al. 2016), small protrusions from neuronal dendrites that form the physical building blocks of memory. These spines begin to provide a method by which to investigate the potential mechanisms of the IRAP inhibitors on memory at a cellular level. Discussed below is a summary of the role dendritic spines play in memory as well as their interaction with IRAP and this will serve as a basis for several other following concepts.

1.4 Dendritic spines as a cellular correlate of memory

One of the most prevalent axioms heard in neurophysiology, particularly within memory, is that "*Cells that fire together, wire together*" taken from Donald Hebb's original postulate "*When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency as one of the cells firing B is increased*" (Hebb 1949). This hypothesis attempted to describe how experiences, transient electrochemical impulses, could be made into lasting structural changes in neurons that allowed memories to be stored within the brain. It has since been accepted that dendritic spines form the basis of these changes, in that sense, our dendritic spines represent the physical form of our memories.

Initially described by Roman y Cajal towards the end on the 19th century, the small spiny protrusions on dendrites in the cerebellar cortex were not identified as sites of synaptic contact until far later with the advent of electron microscopy (Gray 1959). Since then they have been described throughout the brain and are particularly prevalent in the hippocampus. Spines contain all of the synaptic machinery required to form functional synapses and also provide an ideal mechanism to explain many of the predicted mechanism underlying memory and learning

1.4.1 Synaptic communication and spines

Hebbian learning is dependent on the increased synaptic communication between two neurons at the synaptic juncture, also known as LTP. Most simply, LTP describes the ability of the presynaptic neuron to evoke a greater response in the receiving neuron following repeated activation of the two. LTP has become one of the most studied aspects of neuronal communication regarding memory and strong evidence exists to support its role in the mechanism underlying memory with learning of tasks able to promote enhanced LTP (Nabavi et al. 2014). The question that follows is how is this enhanced communication between neurons achieved?

While there are presynaptic mechanisms such as increase in neurotransmitter release, postsynaptic modifications, particularly an increase in

the number of AMPA receptors, are also crucially important.. As spines form the postsynaptic contact for the vast majority of excitatory connections within the brain (Kasai et al. 2010), an increase in their size allows for insertion of more AMPA receptors into the membrane, in turn facilitating an increase in synaptic transmission. Indeed LTP that is induced both chemically or by direct electrical stimulation of neurons leads to an increase in the size of the spine head (Buchs et al. 1996) and stabilization of the overall spine (De Roo et al. 2008).

1.4.2 Spine morphology and memory

In order to respond to novel inputs spines must be highly motile and plastic, able to modulate their shape and signaling rapidly, a property afforded to them by the high content of actin they contain, indeed being the most actin-rich structures in the brain (Fischer et al. 1998). While there a variety of different spine types this review covers the two most relevant to memory, thin and mushroom spines, represented by the blue and yellow arrows respectively in Figure 1-3

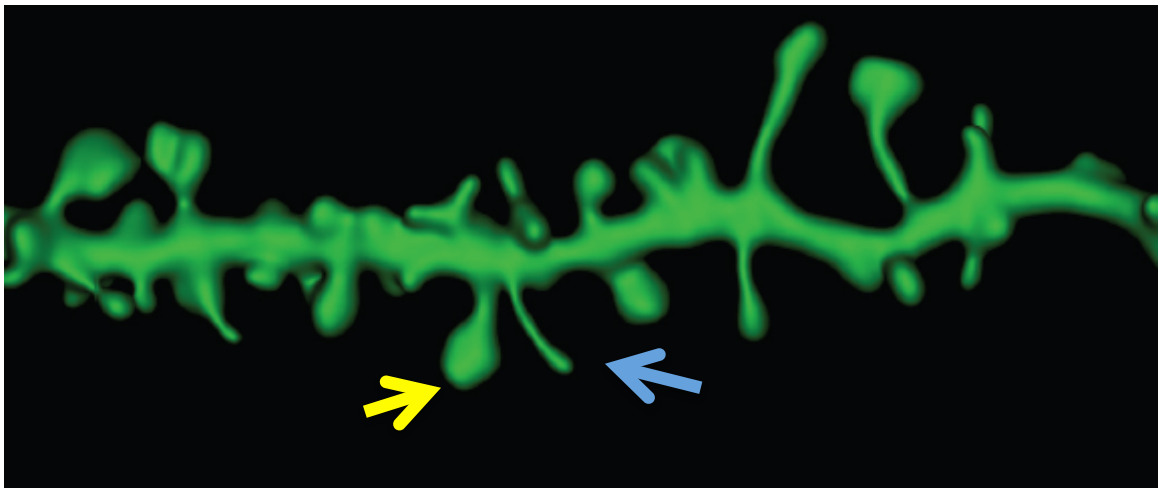


Figure 1-3 3D reconstruction of thin and mushroom spines along a section of a neuronal dendrite.

Thins spines (blue arrow) are characterized by a long neck and relatively small or no swelling. Mushroom spines (yellow arrow) have a shorter neck and large head that contains processing machinery necessary for synaptic communication. Image taken from (De Roo et al. 2008)

The physical differences between the spines are also reflected biochemically. Thin spines have small post-synaptic densities and contain a high proportion of NMDA receptors. On stimulation of the spine these receptors cause an influx of calcium, which triggers a cascade of intracellular signaling leading to the eventual insertion of AMPA receptors into the plasma membrane at the post-synaptic density. To accommodate these extra receptors, the post-synaptic density must grow in size and with it the spine head and it this growth that leads to the transition from thin to mushroom spine.

1.4.3 Dendritic spine density correlates with memory

While the morphology of single spines suggests a mechanism of memory storage, the density of spines along the dendrite also speaks to the cognitive status of the individual. A positive correlation between spine density and learning has been demonstrated in a number of behavioral studies using different paradigms such as passive avoidance (O'Malley et al. 1998), water maze tasks (Moser et al. 1994, O'Malley et al. 2000), fear conditioning (Lai et al. 2012) and repetitive motor tasks (Fu et al. 2012).

While also increasing in response to training, spine number can also be predictive of memory performance. Aged macaques (which had fewer spines) performed worse on a recognition task compared to younger animals (which had a greater number of spines) (Dumitriu et al. 2010). Enriched environments, such as cages with toys and running wheels, have also been demonstrated to increase spine density with rats reared in these environments also performing better on spatial memory tasks such as the water and radial arm maze (Leggio et al. 2005).

1.4.4 Spines in disease states

Just as there is a close positive correlation between cognitive status and dendritic spine density, diseases that result in impairments of memory are also associated with reductions in spine number. This review will restrict itself to the discussion of Alzheimer's disease although this is seen across a number of neurological conditions including schizophrenia and traumatic brain injury.

Loss of synapses is well characterized in AD across several brain regions. Notably, there is a reduction in the hippocampus of Down's Syndrome patients and further reduction in those that also developed AD (Ferrer et al. 1990). This was also seen in AD patients without Down's Syndrome (Ji et al. 2003) and is seen not only in the hippocampus but also multiple cortical layers (Davies et al. 1987, Catalá et al. 1988) and within the temporal lobe (Scheff et al. 1993). The close association between spines and memory is exemplified even further here as synaptic loss forms a better marker of impairment than either plaque or tangle load (DeKosky et al. 1990, Terry et al. 1991, Blennow et al. 1996).

Several transgenic mouse lines of Alzheimer's have also been examined in regards to spine density. These animals express human mutations in amyloid precursor protein either alone or in combination with mutations in presenillin 1 and/or tau. The Tg2576 model demonstrates an impairment in behavioral tasks as well as LTP as early as 4 months of age, correlating with the beginnings of synaptic loss in the model, notably, plaques do not begin to appear before 15 months (Jacobsen et al. 2006). A similar reduction was also observed in another model, the PDAPP mouse, again before the appearance of plaques (Lanz et al. 2003), a finding seen in a number of other models (Rutten et al. 2005, Knafo et al. 2009, Perez-Cruz et al. 2011).

1.4.5 IRAP inhibition is able to increase dendritic spine density

There is a tight correlation between cognitive capacity and dendritic spines, both in terms of their number and morphology. This is particularly relevant to the current work, with inhibition of IRAP already demonstrated to have positive effects on dendritic spine number. Stable analogues of AngIV are able to enhance dendritic spine density in hippocampal cell cultures (Benoist et al. 2011, McCoy et al. 2013), a result replicated with inhibitors designed to mimic vasopressin and oxytocin (Diwakarla et al. 2016). While these results add further evidence to the cognitive enhancing potential of the IRAP-Is, it has not yet been shown that the same inhibitors that enhance cognition are also able to increase dendritic spine growth. Further, the mechanism that underlies this increase in spine growth is still unknown. Considering now the ability of the

IRAP-Is to enhance cognition and dendritic spine density there are several possible mechanisms that could influence both of these factors.

1.5 Mechanisms underlying memory enhancement by IRAP inhibitors

As discussed, IRAP is a complex protein with pleotropic effects. It consists of both an extracellular catalytic domain as well as an intracellular domain with motifs for both signaling and trafficking, which has meant that even now the precise physiological role of IRAP has remained elusive. Given its ability to cleave neuropeptides with potential cognitive enhancing properties one of the most straightforward suggestions has been that inhibition of IRAP allows these substrates to accumulate that in turn enhances memory. While there is some evidence to support an increase in the half-life of pro-cognitive neuropeptides, there are several points to consider.

The majority of IRAP is sequestered intracellularly under basal conditions only moving to the cell surface on stimulation. In neuronal cells, particularly those within the hippocampus, the stimulus for translocation is likely to be membrane depolarization. In this scenario, placing rats in a memory arena will cause an activation of the hippocampus, leading to IRAP translocation to the cell surface where it can cleave neuropeptides such as vasopressin. The inhibitors could then block this action, leading to neuropeptide accumulation and an enhancement of memory. There is some evidence to support this theory given the results that treatment with an oxytocin antagonist is able to ameliorate the pro-cognitive effects of AngIV treatment (Gard et al. 2012), however it is not clear if this is a specific effect. While the hippocampal cells do have receptors for both oxytocin (Insel 1992) and vasopressin (Tribollet et al. 1999), the level of local production of each hormone is unclear. Another caveat here is that the accumulation of the substrates would take time, likely longer than the length of the test in paradigms such as the spontaneous alternation task in which the inhibitors have shown success at enhancing memory.

Further, considering the evidence demonstrating an enhancement of dendritic spine density in primary neuronal cell culture, the substrate hypothesis does not match well here either. While neuronal cells within the hippocampus may express receptors for neuropeptides such as vasopressin and oxytocin there is no evidence that these cell also produce it locally. Thus, in isolated neuronal cultures there is likely no accumulation of the substrate to

account for the increase in dendritic spine density. This leaves two likely mechanisms of IRAP inhibitor-mediated cognitive enhancement that will be discussed below.

1. Enhanced activity of matrix metalloproteinases
2. Increased neuronal glucose uptake

1.6 Matrix metalloproteinases

IRAP has been demonstrated to modulate the activity of MMPs in several physiological settings, within the aorta for example (Numaguchi et al. 2009, Numaguchi et al. 2010). The association of MMPs and plasticity within brain therefore suggests another potential pathway by which the IRAP-Is could enhance memory and learning. MMP-9 is of particular interest here as MMP-9 mRNA and protein show high expression in the hippocampus; particularly the pyramidal cell layer of CA1 and 3, as well as the dentate gyrus. In contrast with MMP-2, which is highly expressed in astrocytes, MPP-9 is highly expressed in neurons (Szklarczyk et al. 2002, Dzwonek et al. 2004, Fragkouli et al. 2012) and upon stimulation MMP-9 mRNA is seen to translocate from the soma to dendrites in cultured cells (Dziembowska et al. 2012), allowing it to play a part in dendritic spine remodeling.

1.6.1 Enhancement of memory

Several different paradigms have demonstrated the importance of MMP9 in memory and learning tasks. MMP-9 KO mice are impaired on hippocampal dependent tasks such as context fear learning (Nagy et al. 2007, Hiroyuki Mizoguchi 2010) and novel object recognition task (Hiroyuki Mizoguchi 2010). Intracranial infusion of MMP inhibitors impairs memory; FN-430 attenuated spatial learning (Meighan et al. 2006, Wright et al. 2007). Conversely, transgenic mice with an overexpression of MMP-9 demonstrated improved memory in the novel object recognition task and Morris water maze (Fragkouli et al. 2012). Interestingly, hippocampal independent tasks such as cued fear learning are unaffected by the loss of MMP-9 (Nagy et al. 2007).

Following context fear learning, an upregulation of MMP-9 is noted in the hippocampus and amgydala (Ganguly et al. 2013). Following from this, pro- and active-MMP9 levels are increased after the induction of LTP and infusion of MMP-9 inhibitor can block LTP (Bozdagi et al. 2007). As noted by many studies alteration of MMP-9 activity appears to have preferentially affect late LTP while having no effect on early LTP (Meighan et al. 2006, Bozdagi et al. 2007, Nagy et al. 2007, Wang et al. 2008, Fragkouli et al. 2012). Indeed infusion of active MMP-9 itself is capable of producing late LTP (Szklarczyk et al. 2002, Nagy et al. 2007,

Wang et al. 2008), and inducing increases in spine volume (Wang et al. 2008), while MMP-9 over-expressing mice show increased spine density in CA1 (Fragkouli et al. 2012).

1.6.2 Mechanisms underlying MMP-mediated plasticity

Two inter-related methods have been proposed by which MMP-9 activity is regulating synaptic plasticity. The first is by allowing structural remodeling; releasing the spine from the local binding of the membrane allows alterations of the spine shape and volume, both necessary for the induction and consolidation of LTP. However, while this theory has been proposed, particularly regarding spine dynamics in the visual cortex (Mataga et al. 2004, Oray et al. 2004), direct evidence of this mechanism in regards to the action of the MMPs is lacking and is also difficult to dissociate from the mechanisms discussed below.

The second proposed mechanism is that cleavage of the extracellular matrix exposes cell surface receptors, such as integrins, and facilitates their activation. The integrins are a broad family of transmembrane spanning glycoproteins and are crucial for the stabilization of LTP (Chun et al. 2001, Chan et al. 2006). These receptors recognize extracellular matrix proteins such as collagen or laminin but can also recognize short sequences within larger proteins, such as the arginine-glycine-aspartate (RDG) binding motif that is found on many extracellular matrix proteins (Aplin et al. 1998). The cytoplasmic tails of these receptors is linked to the actin cytoskeleton (van der Flier 2001) and stimulation can in turn cause reorganization of the cytoskeleton (Chan et al. 2003, Shi et al. 2006). While being a diverse family of receptors, multiple subunit types are enriched in the hippocampus and are localized to dendritic spines (Einheber et al. 1996, Nishimura et al. 1998, Chan et al. 2003, Shi et al. 2006). While several different subunits are expressed at hippocampal synapses they appear to have differential effects on LTP, knockout of the α3 subunit impairs late LTP whereas knockout of either α5 and α8 had no effect (Chan et al. 2003).

Similar to the effects seen when MMP-9 activity is inhibited, inhibition of integrins has no effect on early long-term potentiation but blocks late LTP from developing (Staubli et al. 1990). More directly, late LTP produced by the administration of MMP-9 to hippocampal slices is blocked by the inhibition of

integrin activation (Wang et al. 2008). Exogenous addition of active MMP-9 is also able to cause an increase in phosphorylation of cofillin, which in turn reduces the degradation of the actin cytoskeleton in the spine head and permits enlargement and late LTP (Wang et al. 2008)

Here we can see a potential pathway develop by which the MMPs are able to modulate plasticity. Neuronal activation via the NMDAR induces the expression of nitric oxide, which is able to cleave pro-MMP-9 and activate it, allowing it to degrade the extracellular matrix surrounding the activated spine. While allowing space for the potentiated spine to grow into this also triggers the activation of the integrin receptors that signal intracellularly to increase the growth of the actin cytoskeleton in the spine head.

1.6.3 Summary of MMP-mediated memory enhancement

While the activity of the MMPs appears to be critically important for late LTP, blockade has no effect on early LTP. This means that while increased activity of the MMPs could be promoting increased dendritic spine growth in order to store memories, relevant for tasks such as the novel object recognition, it is not yet clear how the IRAP-Is are able to enhance working memory in paradigms such as the spontaneous alternation task. Nonetheless, the ability of the IRAP-Is with modulating MMP activity in the periphery makes it a compelling avenue of investigation.

1.7 Glucose and the brain

As one of the proposed mechanisms for IRAP-mediated enhancement of memory is the stimulation of glucose uptake into neurons, it is important to give an overview of neuronal metabolism. Currently there is a controversy in the literature as to which of the common sugars, glucose or lactate, form the primary substrate to provide neurons with energy.

1.7.1 Overview of brain metabolism

In the canonical view of neuronal metabolism, neurons are nearly entirely dependent on a constant supply of glucose for survival; as such they express both the constitutive glucose transporter GLUT3 but also the inducible form GLUT4 in order to rapidly increase their glucose uptake when needed. From here, glucose is broken down in the typical manner associated with glucose metabolism, glycolysis to pyruvate followed by oxidative phosphorylation (Figure 1-4).

The alternate pathway proposed is the astrocyte-neuron lactate shuttle hypothesis (ANLSH), which suggests that astrocytes produce lactate that is shuttled across to the neurons where it is converted to pyruvate and enters the TCA cycle (Figure 1-5). Below I will discuss the evidence both for and against lactate as the primary neuronal energy substrate.

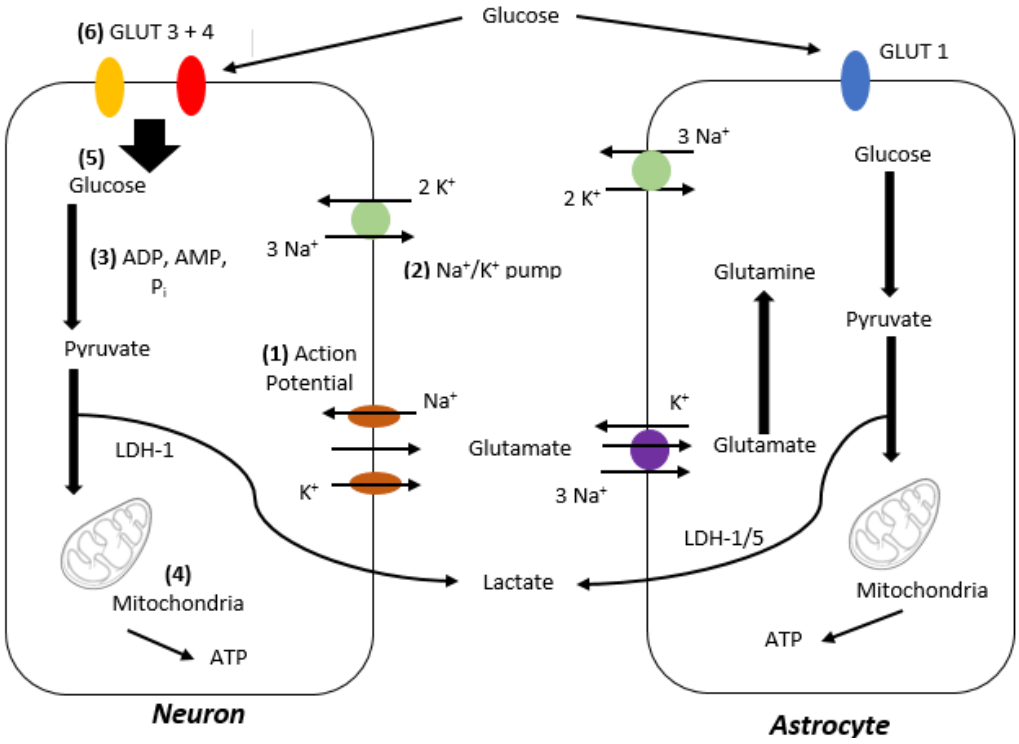


Figure 1-4 Pathway of neuronal activity leading to increased ATP production derived from glucose.

Image adapted from (Wang et al. 2008)

(1) Action potentials causes the influx of sodium of ions and efflux of potassium

(2) To restore homeostasis, the Na^+/K^+ ATPase pump is activated, which consumes ATP

(3a) Increasing ADP, AMP and P_i, as well as falling ATP, trigger glycolysis

(3b) The mitochondria also begin oxidative phosphorylation via the TCA cycle

(4a and b) This drives ATP synthesis, with the majority being generated from the mitochondria

(5) These processes (glycolysis specifically) consume glucose

(6) This in turn triggers GLUT3 to increase uptake into the cell

In this model of brain metabolism, each of the neurons and astrocytes largely supply their own energy derived from glucose. In the neuron this is mainly to power the Na+/K+ pump, while in the astrocytes it also fuels the conversion of glutamate to glutamine.

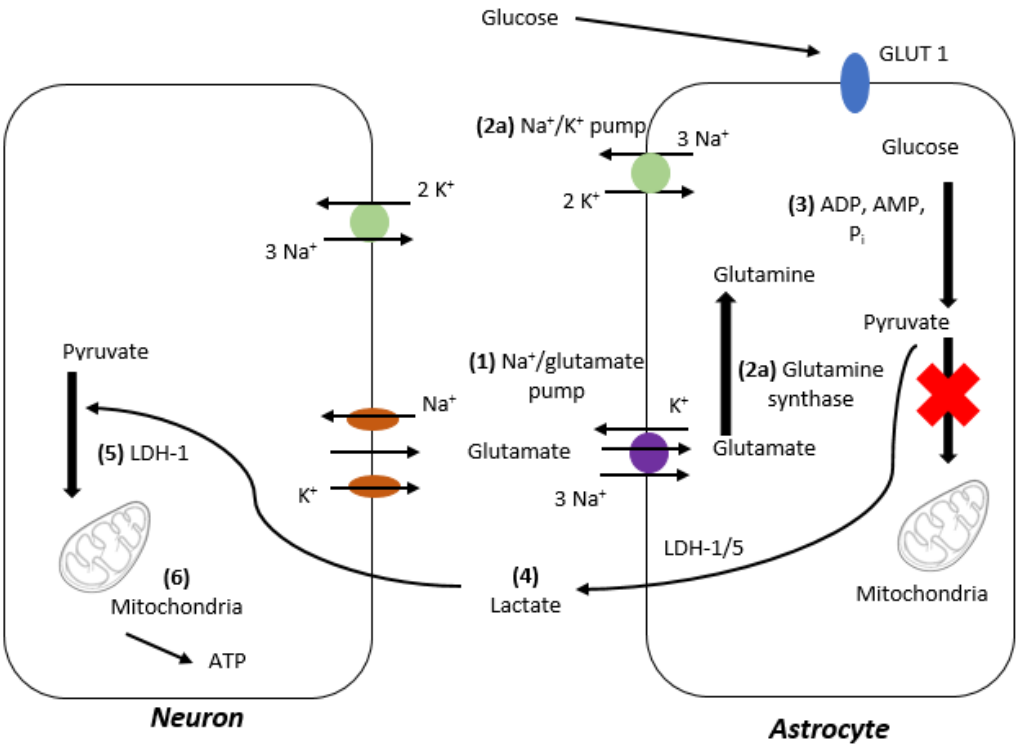


Figure 1-5 Pathway of neuronal activity leading to increased ATP production derived from lactate.

Image adapted from (Wang et al. 2008)

(1) Following an action potential astrocytes activate the Na^+/glutamate transporter

(2a) This activates both the Na^+/K^+ ATPase pump, and

(2b) Glutamine synthase that consumes ATP and increase ADP, AMP and P_i

(3) This activates glycolysis within the astrocytes

(4) Which produce lactate that is then moved into the extracellular space to be taken up by neurons

(5) LDH-1 then converts the lactate into pyruvate

(6) The pyruvate is then able to enter the TCA cycle and undergo oxidative phosphorylation

This model suggests that energy production in neurons is derived exclusively from lactate produced by glycolysis in astrocytes.

1.7.2 Evidence for lactate as the primary neuronal fuel

Some of the first clues to suggest an alternate energy source for neurons came from work correlating glucose and oxygen use throughout the brain. Under resting conditions nearly all of the glucose is oxidized, which would predict a CMRO2/CMR_{glc} ratio of 6 (6 O_2 are required for every glucose). Based on oxygen consumption measurements this would predict that glucose utilization at rest should be around 26.6 mmol/100g of brain tissue, yet actual utilization is around 31 mmol/100g brain tissue (Kety et al. 1948). During neuronal activation the ratio actually falls further, an effect seen in both rats (Madsen et al. 1998) and humans (Madsen et al. 1995). This indicates that not all of the glucose is being fully oxidized, instead undergoing glycolysis, an eventual by-product of which is lactate. The ANLSH predicts that this glycolysis is being performed in astrocytes, which then shunt the lactate across to the neurons as an energy substrate.

More evidence for the ANLSH comes from the transporter expression on neuronal cells. In addition to glucose transporters, neurons also express monocarboxylate transporters (MCTs), which allow the transport of pyruvate, ketone bodies and most importantly in this case, lactate. There are three major classes within the brain (with MCT3 expressed largely in the periphery);

MCT1 is present on endothelial cells, astrocytes and olgiodendrocytes (Bergersen et al. , Gerhart et al. 1997)

MCT2 is a high-affinity isoform is expressed on neurons, predominately in the cerebellum and also the hippocampus where it co-localises with AMPAR subunits at excitatory synapses (Bergersen et al. 2005)

MCT4 in contrast is a low affinity transporter and localized exclusively on astrocytes (Rafiki et al. 2003) and likely helps to mediate transport between the blood and synapses

The basis of the ANLSH postulates that lactate production is intimately linked to neuronal activation. Supporting this, glutamate is able to promote glucose uptake into astrocytes and subsequent release of lactate in astrocyte cultures (Pellerin et al. 1994). Neurons and astrocytes also exhibit different

metabolic profiles with genomic analysis showing that astrocytes contain many genes associated with glycolysis while neurons have a number associated with lactate use (Rossner et al. 2006, Lovatt et al. 2007). In line with this, there is also a predominance of lactate dehydrogenase in astrocytes, while pyruvate dehydrogenase is present in neurons (Bittar et al. 1996, Laughton et al. 2007). This would argue towards an increased emphasis of glycolysis and lactate production in astrocytes as opposed to neurons. Indeed, while both cell types are capable of glycolysis, the rate is faster in astrocytes compared to neurons (Bittner et al. 2010).

Following from this, lactate has been demonstrated to be able to support neuronal function and is indeed protective in detrimental conditions. In cell culture work over-expression of the glucose transporter on astrocytes and over-expression of the lactate transporter on neurons both increased neuronal survival and had a synergistic effect following hypoxia (Bliss et al. 2004). *In vivo*, administration of lactate with reperfusion following ischemic stroke reduced infarct volume and improved neurological outcomes (Berthet et al. 2009)

The evidence discussed so far has not demonstrated a physiological need for lactate; only that it is possible for neurons to utilize it is an energy substrate. Particularly relevant to the current work is that lactate appears to be critical for the formation of memory. Exogenous lactate administration was observed to enhance performance during a spatial memory task, an effect that was abolished by either 1,4-dideoxy-1,4-imino-D-arabinitol (to block lactate efflux from astrocytes) or α-Cyano-4-hydroxycinnamic acid (to block lactate uptake by neurons) (Newman et al. 2011). Expanding on this work, 1,4-dideoxy-1,4-imino-D-arabinitol is seen to have no effect on early LTP, but abolishes late LTP, an effect that correlates well with behavioral work showing no effect on short terms term memory but an impairment of long term (Suzuki et al. 2011). Further, knockdown of MCT4 (which allows efflux of lactate from astrocytes) impaired memory, an effect that could be rescued by the administration of lactate, but not glucose, whereas impairment caused by knockdown of the neuronal MCT-2 was unable to be rescued by either substrate.

1.7.3 Evidence against lactate as the primary neuronal fuel

While there is compelling evidence to support lactate as the primary energy substrate in neurons, there is much evidence to consider against the ANLSH. It is important to consider that substantial evidence to support the ANLSH theory is speculative, arising from the observed expression of various enzymes and transporters rather than direct evidence of metabolism. Though evidence is cited demonstrating that astrocytes are able to; take up glucose (Nehlig et al. 2007), and have a faster glycolytic rate than neurons (Bittner et al. 2010), the fact remains that this same evidence also demonstrates that neurons are indeed capable of taking up and processing glucose. At minimum, this reduces the importance placed on astrocyte-derived lactate as proposed by the ANLSH.

As mentioned, much of the evidence for the ANLSH comes from *in vitro* work though this comes with two major caveats. While the developing brain is reliant on energy sources other than glucose, ketone bodies and lactate being among them, there is dramatic shift towards a glucose preference soon after weaning (Vannucci et al. 2003), meaning that cells harvested from embryos will likely display a phenotype that is not reflective of adult physiology. Recordings of synaptic transmission from hippocampal slices are another common method of studying the effects of altering metabolism. While several studies have reportedly demonstrated the ability of lactate to supplement or even replace glucose in maintaining synaptic activity, it appears that this may only be the case in slowly prepared slice preparations where a shift towards a pathogenic phenotype has occurred (Yamane et al. 2000, Okada et al. 2007). In rapidly prepared slices synaptic activity cannot be maintained with lactate alone. Indeed, when glucose is replaced by lactate these slices maintain adenosine triphosphate (ATP) levels but transmission still falls. This indicates that glycolysis provides some critical element for synaptic activity.

Proponents of the ANLSH also claim that there is a large of amount of evidence to support lactate production by astrocytes and relatively little to support the ability of neurons to utilize glucose. However as summarized eloquently in recent reviews, this is far from accurate, with many labs failing to

reproduce lactate production by astrocytes (Okada et al. 2007) and several demonstrating glucose uptake by neurons (Fernando et al. 2008, Dienel 2012).

There are also kinetic factors to consider when comparing glucose and lactate use by neurons. It has been claimed that lactate is the preferred neuronal substrate for energy in both cells (Bouzier-Sore et al. 2006) and humans with lactate infusion reducing the apparent uptake of glucose into neurons (Wyss et al. 2011). However, glucose metabolism is highly regulated, once hexokinase is saturated, further increases in glucose concentration do not lead to increased use. In contrast, lactate dehydrogenase (responsible for lactate to pyruvate conversion) is unregulated and is merely an equilibrate reaction, thus infusions of lactate unsurprisingly lead to increases in ATP derived from lactate.

The structure of dendritic spines is also critical when considering the energy dynamics of the neuron. Spine heads contain few mitochondria (Li et al. 2004) and as such are unable to utilize lactate. Instead, lactate would have to diffuse down the narrow spine neck, be metabolized in the mitochondria in the dendrite and the resultant ATP would need to diffuse back along the neck into the head before it could be used. The spine neck is narrow precisely to restrict diffusion into the parent dendrite and thus represents an inefficient mechanism of energy metabolism.

While there is still conflict within the literature as to whether lactate or glucose form the primary energy substrate for neurons, the reality is likely predominance towards glucose with lactate supplementing. Indeed, an alternate hypothesis can be proposed when considering the receptor expression on neurons and astrocytes. Under periods of activation spines increase glycolysis in order to rapidly produce ATP. In this scenario it is the neuron that produces the lactate, which then diffuses out of the spine through MCT2 and is taken up by the astrocyte for recycling. Importantly however, as will be discussed below, exogenous glucose administration is able to enhance memory, thus, even if lactate forms the primary energy substrate, this does not argue against a glucose-mediated enhancement of memory in response to IRAP inhibition.

1.7.4 Glucose loading enhances memory

1.7.4.1 Enhancement in rodents

Both peripheral and central loading of glucose enhances memory in rats. Microdialysis of the hippocampus demonstrates that extracellular fluid levels of glucose fall during performance of memory tasks such as the spontaneous alternation maze (McNay et al. 2001) and that systemic administration of glucose prevents this decline while also enhancing memory (McNay et al. 2000). Indeed, aged rats (24 months old) demonstrate impaired memory compared to young rats (3 months old) as well as greater reductions in extracellular glucose levels in the hippocampus. Meanwhile, peripheral administration of glucose ameliorated both memory and hippocampal glucose levels in the aged animals (McNay et al. 2001). In addition to an enhancement in spatial tasks, peripheral glucose administration shows positive effects on cognition in area habituation (Kopf et al. 1996), operant conditioning (Messier et al. 1988, Messier et al. 1990) and conditioned avoidance tasks (Kopf et al. 1994)

Central administration of glucose can also enhance memory with intrahippocampal administration enhancing memory in the spontaneous alternation task (Ragozzino et al. 1998). Glucose is also able to rescue induced deficits in memory with delivery into the medial septum able to reverse deficits caused by morphine infusion (McNay et al. 2006).

1.7.4.2 Enhancement in elderly subjects

In humans some of the earliest evidence for the facilitation of memory by glucose ingestion came from work in elderly subjects. Ageing is generally accompanied by neuroendocrine dysfunction, in particular impaired gluco-regulatory control (Messier 2004), as well as hormones related to both memory storage and glucose regulation (Gold 2005). This led to the assumption that glucose supplementation could alleviate the associated cognitive impairments. Indeed, glucose ingestion enhanced verbal episodic memory in healthy elderly individuals (Hall et al. 1989, Allen et al. 1996, Manning et al. 1998, Riby 2004, Riby et al. 2004, Riby et al. 2006) with the most efficacious blood glucose range post ingestion being 8 – 10 mmol/L (Parsons et al. 1992).

Beyond the enhancement of verbal episodic memory, glucose also enhanced attention (Messier et al. 1997, Reay et al. 2006), working memory (Sünram-Lea et al. 2004), semantic memory (Riby et al. 2006), visual memory (Allen et al. 1996) and visuospatial function (Scholey et al. 2002). This enhancement of memory by glucose also appears to be non-specific; enhancing memory whether ingested pre-encoding, post-encoding or pre-retrieval (Manning et al. 1993, Manning et al. 1998).

1.7.4.3 Enhancement in young adults

While there are consistent results within elderly patients, whether glucose administration produces the same effects within young adults is not as clear. Several studies have suggested that glucose does not appear to confer a cognitive benefit in young adults when used in the same memory paradigms as older subjects (Hall et al. 1989, Azari 1991, Manning et al. 1997, Macpherson et al. 2015), however, there are reports of enhancement in young adults (Smith et al. 2009, Scholey et al. 2014). In work designed to directly address this issue it appears that facilitation is most consistently seen in younger adults when more challenging protocols, such as divided attention tasks, are employed (Sunram-Lea et al. 2002, Riby et al. 2006).

This shows that in young subjects who likely have good gluco-regulatory control, or neuronal cells that are simply more efficient at using the available fuel, glucose cannot enhance cognition. However, stressing the system through the use of more difficult task conditions could exhaust the local supply of glucose, thus allowing the glucose loading to again produce a positive effect. Interestingly, exogenous glucose administration also appears to be able to rescue deficits of memory such as those caused by mild head trauma (Pettersen et al. 2000), this is particularly relevant when considering the potential applications in disease states such as Alzheimer's disease.

1.7.4.4 Effect of glucose in AD

Discussed at far greater detail later in this thesis, cerebral hypometabolism is emerging as a distinct and specific phenotype in AD. Given this, a small number of studies have examined the ability of glucose loading to rescue memory deficits present in the disease. Oral glucose administration

improved episodic memory in AD patients (Craft et al. 1992) and (Manning et al. 1993) demonstrated a similar effect with an improvement in verbal episodic memory, a finding replicated by the same group in another study using hyperglycaemic clamping to control blood glucose levels (Craft et al. 1993)

Despite the promising results these studies demonstrate, prolonged glucose loading would naturally lead to issues with gluco-regulation possibly leading to the development or advancement of a diabetic state. As such, development of a therapy that can directly enhance uptake into neurons, such as the proposed mechanism of IRAP inhibitors, offers a potential therapeutic option.

1.7.5 Mechanisms of glucose-mediated memory enhancement

While there is variability in terms of the exact treatment protocol, there is strong evidence to demonstrate the positive effect that glucose loading can have on memory and there are several mechanisms that could be mediating this effect.

1.7.5.1 Peripheral Hormone Release

Peripheral glucose loading leads to an elevation of blood insulin and epinephrine levels, which raises the possibility that these hormones may be mediating the positive cognitive effects seen in response to glucose loading. Many regions of the brain, particularly those critical to memory and learning, such as the hippocampus, show high levels of insulin receptor expression (Young et al. 1980, Marks et al. 1990, Doré et al. 1997), suggesting it plays a role in cognition. Additionally, insulin loading in its own right has been demonstrated to enhance memory learning when administered centrally in rodents (Park et al. 2000) and also intranasally in humans (Benedict et al. 2004, Benedict et al. 2007). In a similar fashion, epinephrine can also enhance memory in both rodents (Gold et al. 1975) and also humans (Cahill et al. 2003), however, this effect appears to be strongest when the memorization task involves emotional arousal.

1.7.5.2 Central Glucose Loading

Two of the potential mechanisms of glucose-mediated memory enhancement rely on hormone secretion (insulin and/or epinephrine) in the periphery before crossing into the brain and having their action. Several studies have examined the effects of direct central administration of glucose, noting that there is still an enhancement of memory when delivered into the ventricles (Lee et al. 1988, Parsons et al. 1992) or directly to the hippocampus (Dash et al. 2006). These studies do not necessarily preclude insulin from still mediating these cognitive enhancements, as there is evidence to show that insulin can be produced locally within the brain. Less than 1% of peripheral insulin enters the brain (Banks et al. 1998), yet brain levels are still relatively high and indeed appear independent from plasma insulin levels (Havrankova et al. 1979), indicating local production within the brain. Supporting this, insulin has been observed to be released from neuronal cells in culture (Clarke et al. 1986).

Even if the cognitive enhancing effects of glucose are indeed mediated by an increase in insulin, either peripheral or central, it is unlikely that this is interacting with the action of IRAP. As we predict that IRAP inhibition enhances glucose uptake directly into neurons, it is far more likely that increased cholinergic release or restoration of a local deficit is playing a role.

1.7.5.3 Restoration of local deficit

Performance of cognitive tasks leads to a fall in extracellular glucose levels as neurons enhance uptake in order sustain their activation. Indeed, the reduction in extracellular glucose is proportional to the difficulty of the task (McNay et al. 2000, McNay et al. 2001). It is possible that this reduction in the availability of glucose forms a rate-limiting step for neuronal activation and thus cognitive activity. Exogenous glucose administration could be compensating for this fall and thus allowing continual peak activity by neurons. However, this mechanism is unlikely to play a role in IRAP-mediated memory enhancement as inhibitors promote increased neuronal uptake, not an increased delivery of glucose.

1.7.5.4 Increased cholinergic Release

Acetylcholine (Ach) is a crucial neurotransmitter for the formation of memory (Hasselmo 2006) and glucose administration can reverse the effects of memory impairment induced by cholinergic blockade (Stone et al. 1991, Micheau et al. 1995). At doses which glucose improves memory, there is enhanced release of ACh in the hippocampus during the performance of behavioral tasks (Ragozzino et al. 1996, Ragozzino et al. 1998). Glucose is a precursor to acetylcoenzyme A that in turn forms a precursor to ACh (Tucek et al. 1982), thus increases in the extracellular concentration of glucose can lead to increased ACh synthesis (Durkin et al. 1992), while glucose deficits lead to a reduction in synthesis (Gibson et al. 1976). Importantly this provides a mechanism by which glucose loading can improve working memory, ACh being a key neurotransmitter for this process (Daniel et al. 2001, Green et al. 2005).

Further evidence to support ACh-mediated glucose enhancement is seen in the effect on ATP-sensitive potassium (K-ATP) channels, inwardly rectifying K^+ channels whose closure increases the likelihood of neuronal firing (Inagaki et al. 1995). Increased intracellular ATP (derived from glucose) leads to the closure of the channels, thus providing a mechanism for how glucose administration is able to enhance ACh release. In support of this, injection of galanin, a neuropeptide capable of increasing K-ATP conductance (De Weille et al. 1988), impairs both memory and cholinergic transmission (Hiramatsu et al. 1996), an effect reduced by administration of both glucose and glibenclamide (a K-ATP channel blocker) (Stefani et al. 1998). Indeed glucose and glibenclamide are able to act synergistically to enhance memory (Stefani et al. 1999).

1.7.5.5 Activation of mTOR

Glucose can also activate mammalian target of rapamycin (mTOR), a regulator of cell growth of proliferation (Dash et al. 2006). In turn, mTOR drives an increase in protein synthesis, a key component for dendritic spine growth and L-LTP (Dong et al. 2003) and inhibition of these processes inhibits long term memory formation (Dash et al. 2006, Jobim et al. 2012, Mac Callum et al. 2014). The ability of glucose to activate mTOR is particularly relevant as it shows the pathway that glucose loading would be able to enhance long-term memory.

1.7.6 Glucose Transporters

The brain uses more glucose per mass than any other organ of the body and neurons themselves account for 85% of this usage, as such there are numerous specialized glucose transporters throughout the brain to meet this demand.

1.7.6.1 GLUT1

There is abundant GLUT1 expression on all blood vessel endothelial cells throughout the brain (Harik et al. 1990) and also on the surface of astrocytes (Leino et al. 1997), but not on neurons. This placement ideally allows GLUT1 to transport glucose across the blood brain barrier from the circulation.

1.7.6.2 GLUT2

The low-affinity GLUT2 transporter likely functions as a glucose-sensor within the brain given its ability to transport glucose across a wide-range of concentrations. It is also localized in a number of regions in the brain where glucose-sensing is known to occur and has been shown to be expressed in a small proportion of both astrocytes and neurons (Leloup et al. 1994, Arluison et al. 2004).

1.7.6.3 GLUT3

GLUT3 is expressed exclusively by neurons (Maher 1995) and is localized primarily to the neurophil (McCall et al. 1994) and in particular to the pre- and post-synaptic sites (Leino et al. 1997). As such, GLUT3 is in prime position to allow glucose uptake into neurons at the sites of highest activity, namely the dendritic spines and the high-affinity, high-activity of the transporter argue for its role in supplying neurons with glucose for energy (Maher et al. 1996).

1.7.6.4 GLUT8

Also known as GLUTx1, GLUT8 appears to be expressed exclusively by neurons, particularly within the hippocampus (Ibberson et al. 2000) and in contrast to GLUT3, only at the cell body (Reagan et al. 2002), suggesting its primary role is to meet the global energy requirements of the neuron. However, insulin stimulation of primary hippocampal cultures resulted in translocation to

the endoplasmic reticulum rather than the plasma membrane (Piroli et al. 2002) casting doubt on this proposed mechanism.

1.7.7 The interaction of IRAP and GLUT4

GLUT4 shows high levels of expression in several regions including the cerebral cortex, basal ganglia, hypothalamus, thalamus, amygdala and hippocampus (Apelt et al. 1999). In peripheral tissues GLUT4 is highly insulin responsive, translocating to the plasma membrane on stimulation of cardiomyocytes, adipocytes and skeletal muscle cells. Whether neurons, particularly hippocampal neurons, share this property is currently debated, however within the hypothalamus GLUT4 likely plays a role in glucose sensing (Ren et al. 2014, Ren et al. 2015) while in the hippocampus it could be involved in memory and learning (Wang et al. 2014).

1.7.7.1 IRAP and GLUT4 in peripheral tissues

As discussed earlier, IRAP plays a key role in regulation of GLUT4 vesicle trafficking. Given that these two proteins bear a very close association within vesicles it is unsurprising that modulation of GLUT4 alters the expression and distribution of IRAP and in turn modulation of IRAP affects GLUT4, although the precise consequences are currently unclear.

In cells taken from GLUT4 knockout mice, adipocytes had increased IRAP expression while cardiac and skeletal muscle showed decreased expression (Jiang et al. 2001). This reduction in expression in cardiac tissue is specific to IRAP and is not related to generalized issues with vesicle transport (Abel et al. 2004). In contrast to the study by Jiang and colleagues, siRNA knockdown of GLUT4 in 3T3-L1 adipocytes led to a reduction in IRAP expression (Yeh et al. 2007). The variation in results could be due to the differential timing and global vs. specific effects, whereby knockdown of GLUT4 before adipocyte differentiation can affect IRAP expression whilst it has little effect after development (Liao et al. 2006). Overexpression of GLUT4 produces the opposite effect, an increase in the expression of IRAP (Carvalho et al. 2004). It has been suggested that both IRAP and GLUT4 are required for correct targeting to glucose storage vesicles (GSVs) or that they are degraded by the same pathway, thus lack of one leads to an enhanced clearance of the other. This hypothesis is

supported by the lack of change in IRAP mRNA levels in GLUT4 knockouts which suggests post translational changes (Abel et al. 2004)

The results seen in the GLUT4 knockout mice are largely paralleled by those seen in the IRAP KO mice. In both adipocytes and skeletal muscle GLUT4 expression is reduced as is recruitment to the plasma membrane during insulin stimulation (Keller et al. 2002). Interestingly however, the relative distribution of GLUT4 between the plasma membrane and cytsol is unchanged and the IRAP KO mice show no difference in basal serum glucose levels or in response to an oral glucose tolerance test. This does suggest that IRAP may not play a role in the targeting of GLUT4 to the correct vesicles. However, injection of a truncated cytoplasmic tail portion of IRAP is able to increase glucose uptake in adipocytes (Waters et al. 1997), presumably by disrupting the endogenous IRAP tethering of the GSVs within the cell causing them to translocate to the plasma membrane.

One of the major limitations when speculating about the action of IRAP and glucose uptake is that a large proportion of the work discussed has been conducted in peripheral tissues, namely the heart, skeletal muscle and adipocytes. IRAP and GLUT4 do traffic and co-localize very well in these tissues and cells, but skeletal muscle and adipocytes in particular are highly insulin responsive, while neuronal cells are far less so, even unresponsive as demonstrated by our own previous work (Fernando et al. 2008) and that of others (Goodner et al. 1980, Orzi et al. 2000). This makes it difficult to observe the relationship between IRAP and GLUT4 that we see in peripheral tissues and apply it directly to neuronal cells. This does not mean that the interaction between the two proteins is unable to increase glucose uptake, rather, different forms of stimulation, such as neuronal activation may be required to cause translocation.

1.7.7.2 IRAP, GLUT4 and glucose uptake within the brain

Similar to peripheral tissue, IRAP and GLUT4 share a close association in both the mouse and rat brain (Fernando et al. 2005, Fernando et al. 2007), particularly in the hippocampus where nearly all GLUT4 positive cells are IRAP positive (Fernando et al. 2008). This co-localisation is present at the sub-cellular level with both proteins having punctate staining characteristic of GSVs and

enrichment in the low density micro fraction with a small proportion present at the plasma membrane (Fernando et al. 2007).

Beyond this close association between the two proteins, inhibition of IRAP is able to enhance glucose uptake in hippocampal neurons. In hippocampal slices, treatment with either the peptide inhibitors (Fernando et al. 2008) or the synthetic inhibitor, HFI-419 (Albiston et al. 2008) was able to increase glucose uptake. This effect was not seen in cerebellar neurons where there is little co-localisation between IRAP and GLUT4. Importantly, both IRAP and GLUT4 are required for this effect, glucose uptake could not be potentiated in slices taken from IRAP KO or those treated with indinavir (a GLUT4 inhibitor) (Fernando et al. 2008).

Speculating as to the mechanism of enhancement, the inhibitors could be increasing uptake by prolonging the amount of time that GLUT4 and IRAP remain at the cell surface. In adipocytes IRAP returns to the intracellular pool from the plasma membrane within 2 minutes, indicating a constant recycling (Ross et al. 1997). As IRAP and GLUT4 are required to move together in order for correct intracellular processing, the binding of the inhibitors could disrupt the recycling process and thus maintain both proteins at the plasma membrane, in turn increasing glucose uptake. There is evidence to suggest that inhibitors can induce conformational changes in the structure of IRAP (Mpakali et al. 2017), though this has only been demonstrated for the catalytic site as yet. Both the peptide and synthetic inhibitors were only able to promote glucose uptake in stimulated neuronal cells (Albiston et al. 2008, Fernando et al. 2008), which is consistent with the proposed theory and indicates that they can only modulate the activity of IRAP once it is at the cell surface.

One note is that both IRAP and GLUT4 are found in the cell body, rather than the dendrite processes as GLUT3 is (Fernando et al. 2007). Localization to the dendritic spines would be expected in order to provide increased glucose upon stimulation of the spines to provide energy for spine enlargement for maintenance of L-LTP. This does not necessarily preclude IRAP inhibition from acting through increased glucose uptake, it is possible that by increasing the

glucose uptake into the cell there is an overall increased energy budget associated with the cell allowing it greater ability to undergo plasticity.

1.7.8 Summary of glucose-mediated memory-enhancement by IRAP inhibition

The most compelling evidence to support this hypothesis is the timescale of action. Whereas substrate accumulation likely takes longer than the length of some of the tasks, and MMP expression is required for the consolidation of long-term memories, neither can adequately account for the improvement in working memory caused by the inhibitors. This requires a rapid and immediate effect; one that increased glucose is ideally placed to meet. As discussed earlier, increased glucose uptake into the cell could either increase the likelihood of neuronal depolarization due to the interaction with the K-ATP channel or lead to the formation of more ACh. In turn, both of these actions could enhance working memory. The activation of mTOR by glucose also provides a pathway for the facilitation of long-term memory and could explain the improvement in long-term memory seen in response to IRAP inhibitor treatment in tasks such as the novel object recognition or passive avoidance.

1.8 Summary of possible mechanisms for IRAP-inhibitor mediated cognitive enhancement

1.8.1 Substrate accumulation

The extracellular catalytic domain of IRAP is predicted to cleave a number of neuropeptides that have pro-cognitive effects, thus inhibition of IRAP would allow these substrates to accumulate and promote increased memory and learning. Several lines of *in vitro* evidence do show the ability of IRAP to cleave these substrates and there is some *in vivo* evidence in support this hypothesis. However, the timescale of these effects cannot explain all of the memory enhancements nor the cell culture results. Further, there is no evidence that these substrates accumulate *in vivo* in response to IRAP-I treatment.

1.8.2 Enhanced MMP activity

In contrast to the substrate hypothesis, there is demonstrated *in vitro* and *in vivo* evidence that IRAP inhibition can differentially affect the expression and activity of various MMPs. This could promote increased matrix cleavage, allowing for greater dendritic spine growth, a crucial factor consolidating memory. However, this again cannot explain all of the observed pro-cognitive effects of IRAP inhibition, particularly the enhancement of spatial working memory. In addition, there is currently no confirmed pathway that links the inhibition of IRAP with an alteration in MMP.

1.8.3 Increased glucose uptake

The glucose uptake hypothesis has the most compelling evidence to support it as the mechanism of cognitive enhancement underlying IRAP inhibition. The close association of IRAP with GLUT4, as well as several tethering proteins, makes it aptly placed to increase glucose uptake. Several *in vitro* experiments have demonstrated the ability of the both the peptide and synthetic IRAP-Is to enhance stimulated glucose uptake in brain slices. Increased glucose uptake can also explain both the immediate improvement of working memory by providing the cell with an increased energy budget while also stimulating a number of downstream pathways to enhance dendritic spine growth.

In addition to the investigation of the mechanism that governs the effects of the IRAP-Is, there is also interest as to their potential use in diseases that feature a cognitive impairment. Alzheimer's disease is of particular interest given that it features cognitive impairment, a reduction in spine density and is also characterized by a specific pattern of cerebral glucose uptake deficits. This suggests IRAP-Is may have particular potential in the treatment of the disease given their suggested ability to modulate all three of these parameters. Further, IRAP activity correlates with cognitive status in AD (del Carmen Puertas et al. 2013), although this has been disputed by others (Gard et al. 2017).

1.9 Current consensus regarding AD

1.9.1 Neuropsychological impact of AD

While there are a variety of histopathological features associated with AD, definitive diagnosis is always contingent on the presence of memory impairment first (Dubois et al. 2010). Indeed, any other factors, such as the often-discussed amyloid plaques, form supportive criteria for the diagnosis of the disease Table 1-1.

Core diagnostic criteria	**Features**
Episodic memory impairment	**1. Gradual and progressive change in memory function** **2. Objective evidence of memory impairment on testing** **3. Memory impairment can be isolated or associated with other cognitive changes**
Supportive criteria	
Medial temporal lobe atrophy	• **Volume loss of hippocampi, entorhinal cortex, amygdala as evidenced on MRI**
Abnormal cerebrospinal fluid biomarker	• **Low $A\beta_{42}$, and/or** • **Elevated tau or phosphor-tau**
Specific pattern on FDG-PET	• **Reduced glucose metabolism in the bilateral temporal parietal regions**
Hereditability	• **Proven AD autosomal dominant mutation within the immediate family**

Table 1-1 - Diagnostic criteria for the diagnosis of probable Alzheimer's disease

Tracing the precise timeline of memory impairment is far more difficult than following pathology progression, largely because cognitive tests are only able to specifically test for given domains of memory and it can difficult to conclusively determine if these result from pathological damage. For example, subjects can perform poorly on tests of executive function (control of behaviour)

if they have impaired semantic memory (concept formation) (Kivisaari et al. 2012). Likewise, difficulties with attention make will affect the performance on nearly all tasks.

The most characteristic component of cognition affected in AD is episodic memory (Benjamin et al. 2015), information about autobiographical events and past experiences. This impairment is typically associated with deficits in the medial temporal lobe and is seen across a range of memory types including odour (Dhilla Albers et al. 2016), auditory (Becker et al. 1996) and visual (Meyer et al. 2016) recollection. These problems appear to be due to both issues with encoding and storage of information (Delis et al. 1991) and also retrieval. Highlighting the issues discussed previously, this impairment may be due to increased interference from previously learned memories (Jacobs et al. 1990) as well as an impaired ability to use semantic information to aid with encoding (Crocco et al. 2014). This overall impairment to semantic memory is also a key feature of AD (Hodges et al. 1995, Hirni et al. 2013). Working memory, the ability to hold and use information in the mind is also affected in the course of the disease, most typically presenting as an impairment attending to multiple simultaneous tasks (Amieva et al. 2008, Wilson et al. 2011).

Typically associated with pathology spread to association cortices of the brain, several other facets of cognitive function are also impaired. This spread to the temporal, frontal and parietal lobes leads to an impairment of executive control (Godefroy et al. 2016) as well as language fluency (Rodríguez-Aranda et al. 2016) with both forgetting of words and also intrusion of errors (Gollan et al. 2017). Notably, as discussed below, the relative sparing of motor and sensory cortices is reflected in the ability of AD patients to still able to learn procedural motor tasks (Hirono et al. 1997, Chauvel et al. 2017).

1.9.2 Neuritic plaques

The deposition of amyloid plaques, which are composed of aggregated amyloid-β peptides, either 40 or 42 amino acids in length, is arguably the most researched aspect of AD and provides the basis for the majority of animal models. (Figure 1-6) describes a simplified pathway by which the amyloid precursor protein (APP) is cleaved to form the aggregate-prone peptides.

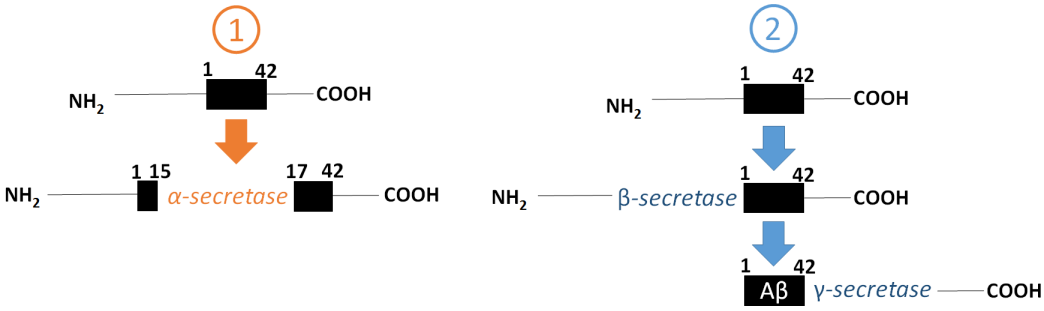


Figure 1-6 APP molecule degradation pathway in Alzheimer's disease

In normal processing (1), the APP molecule is cleaved in the middle of the transmembrane portion by α-secretase and this releases a large soluble ectodomain fragment into the extracellular space. APP molecules that are not cleaved in this way undergo a secondary pathway described in (2). Here β-secretase cleaves closer to the NH_2 terminal, releasing a smaller ectodomain fragment. The remaining portion is then acted on by γ-secretase that can yield either a 40 or 42 amino acid fragments with the 42-length form considered more pathogenic.

Evidence for the role of amyloid as the causative agent for AD begin with observations of individuals with Down's syndrome, a genetic condition that features a triplicate copy of chromosome 21, on which APP is located. Down's syndrome is associated with the development of early AD neuropathology suggesting a gene dosage effect attributed to *APP* (due to overproduction of *APP* resulting from the extra copy) (Holland et al. 1998). Support for this comes from the case of an individual who had partial trisomy of chromosome 21, but lacking the region-containing APP. While this person displayed features typical of Down's Syndrome, they did not develop cognitive decline normally associated with the disease in middle age nor did they present with significant Aβ pathology on autopsy (Prasher et al. 1998).

Familial Alzheimer's disease supports the pivotal role of amyloid in AD pathogenesis. Mutations in *APP* lead to the increased production of the pathogenic species of Aβ (Aβ42) and an earlier onset of the disease (Nilsberth et

al. 2001, Theuns et al. 2006). In a similar fashion, mutations in *PSEN1* can lead to increased activity of γ-secretase that in turn increases the production of Aβ42 (Ikeuchi et al. 2003). Given the inherent difficulty in studying the pathogenesis of AD in humans, the majority of research has focused on several genetic mouse models of the disease that feature insertions of these same mutated genes that have been identified in humans. In this way they provide a model in which to test aspects of the amyloid hypothesis. The three mutations that are relevant to the mouse models that will be discussed later are;

- The London mutation (V717F) that features a valine to phenylalanine mutation at position 717 in the APP gene (Goate et al. 1991). This causes an increase in the production of the more amylodogenic Aβ42 (Eckman et al. 1997)
- The Swedish mutation (K670N/N671L) contains a double mutation with a leucine to asparagine substitution at position 670 and also a methionine to leucine substitution at 671. This leads to a greater production of both Aβ 40 and 42 however the overall ratio is largely unchanged
- The Florida mutation (I716V) has a Isoleucine to valine mutation at position 716 leading to an increase in the production of Aβ42 (De Jonghe et al. 2001)

However, even in humans, amyloid deposits do not appear as predictably as other features, such as tau tangles. There is a general pattern involving deposition first in regions of the neocortex that comprises the "default mode network" (a series of heavily connected brain regions most active during undirected thought). From here the deposits progress into the allocortex (particularly the hippocampus, amygdala and cingulate cortices). One criticism leveled against amyloid being the prime causative agent of AD is that episodic memory is one of the earliest cognitive factors to be affected (suggesting large hippocampal involvement), however amyloid deposition begins in the cortical regions. A counter argument to this has been that due to the default mode networks heavy projections to the medial temporal lobe, dysfunction in these areas leads to an impairment of memory (Buckner et al. 2005)

The third stage is characterised by involvement of further subcortical regions including the striatum, thalamus and hypothalamus. In the advanced stages of the disease brainstem structures become involved before the cerebellum is ultimately affected (Thal et al. 2002). Notably, in this progression of pathology, the areas affected are not those that are generally associated with the cognitive symptoms of the disease. Another point of contention is the relationship between disease severity and plaque load, with several studies demonstrating little to no correlation (Gómez-Isla et al. 1997, Giannakopoulos et al. 2003, Ingelsson et al. 2004). Indeed some elderly people may display extensive amyloid deposits yet remain cognitively indistinguishable from those with few to no plaque load (Troncoso et al. 1996). These points highlight that amyloid, while necessary, is not sufficient for AD, a concept that will be discussed below.

1.9.3 Neurofibrillary tangles

The second of the original histopathological features originally defined by Alois Alzheimer, neurofibrillary tangles (NFTs), differ in both composition and progression compared to the amyloid plaques. NFTs are comprised of hyper-phosphorylated tau; a protein normally associated with microtubules of the cell's cytoskeleton and is believed to be involved in cytoskeleton stabilization. The hyper-phosphorylation of tau prevents normal binding of tau with the microtubules and leads to self-aggregation.

Whereas the amyloid hypothesis has strong genetic evidence to support its role in AD, much of the support for tau comes from its close correlation with the clinical progression of the disease. Unlike plaque deposits, tangles begin forming in areas related to the symptoms of the disease. Deficits in episodic memory (formation of new personal memories) are considered the clinical hallmark of AD and thus it is not surprising that the brain area crucial for this, the hippocampus, is among the first to show NFTs (Braak et al. 1991). Following this, other structures within the medial temporal lobe, such as the amygdala, become affected before pathology spreads into the association cortices (Arnold et al. 1991). This high order involvement correlates well with the symptomatic progression of the disease where impairments of executive function and

semantic memory are noted earlier on. Lastly, as expected given the relative lack of symptoms throughout the majority of the disease, the primary sensory areas become affected.

When considering the mouse models, those that rely solely on APP and *PSEN1* mutations notably do not develop NFTs. In order for this phenotype to be displayed a mutation specific to tau is required, the *MAPT P301L* for example, normally associated with frontotemporal dementia in humans (Oddo et al. 2003). Furthermore, despite correlating well with the symptomatic progression of the disease and following the same spatiotemporal pattern as neuronal loss, cell death still exceeds NFT formation (Gómez-Isla et al. 1997), again suggesting that there are other underlying factors contributing to the pathology of AD. This is compounded by the fact that the available drugs for AD do not alter disease progression (O'Brien et al. 2011) and at best treat the symptoms of the disease for a limited time. Therefore, there remains an urgent need for an effective drug therapy. After decades of research into the disease, several drugs have displayed initial promising results in preclinical animal trials, often showing substantial reductions in pathology, however these have subsequently failed in clinical trials (Green et al. 2009, Quinn et al. 2010, Hung et al. 2017). This may indicate that perhaps the models themselves may not be complete.

1.9.4 The interaction of the renin angiotensin system and AD

At this point it is prudent to mention the association between therapeutics used to treat hypertension and the risk of AD. Both the angiotensin converting enzyme inhibitors (ACE-Is) and angiotensin receptor blockers (ARBs) have been associated with a reduced risk of developing AD, independent of their effect on blood pressure.

Patients treated with centrally acting ACE-Is had a slower rate of cognitive decline (O'Brien et al. 2011) and ACE-I treatment increased AB42 levels in the plasma, indicating increased cerebral clearance (Regenold et al. 2017). The ARBs have also been observed to have potentially beneficial effects on several AD markers (Saavedra 2016). In animal models, losartan (an ARB) improved cognition in AD mice (O'Caoimh et al. 2014) and this effect was found to be mediated by increased levels AngIV (Royea et al. 2017). This is in keeping

with previous suggesting that ACE-I and ARB treatment increases the conversion of AngII and AngIV (Braszko et al. 2006). Taken together this provides further evidence for the potential of the IRAP-Is to play a role a potential therapeutic in AD.

1.10 Hypometabolism in Alzheimer's disease

Of particular relevance to the current work given the potential role of IRAP in increasing neuronal glucose uptake is the reduced cerebral glucose utilization (CGU) that is a key feature of AD. Reductions in CGU follow a specific spatio-temporal pattern in the progression of AD and form one of the supporting diagnostic criteria for the disease (Dubois et al. 2014). Evidence highlights a specific spatial and temporal pattern of hypometabolism present within the brain of Alzheimer's patients, with suggestion that it can be used to differentiate AD from other forms of dementia (Mosconi et al. 2008) and predict those at risk of developing the disease (Fouquet et al. 2009). While the human phenotype is well characterised and serves as one of the criteria for diagnosis (Marcus et al. 2014), the phenotype of the Alzheimer's mouse models is currently conflicted within the literature. Differences between the animal models and the human condition may partly explain why drugs showing promise in the animals are not successful in humans. As such, this review seeks to collate the currently published studies on cerebral glucose metabolism in mouse models of AD and compare them to the phenotype seen in humans.

While hypometabolism within the AD brain has been known for over two decades, recent advances in imaging technologies, particularly ^{18}FDG-PET, has allowed much greater investigation into the progression of hypometabolism. Broadly, hypometabolism begins within the parieto-temporal cortices and limbic areas before spreading into the frontal areas, while the primary sensory areas and cerebellum are largely spared (McGeer et al. 1990, Mielke et al. 1996, de Leon et al. 2001, Herholz et al. 2002, Mosconi et al. 2008, Mosconi et al. 2009, Yuan et al. 2010). Considering that the progression of hypometabolism is highly conserved between patients, it now constitutes one of the diagnostic criteria for AD (Dubois et al. 2007). Furthermore, recent evidence suggests that hypometabolism can be used to predict disease risk, with individuals who show larger reductions in cerebral metabolism within the posterior cingulate cortex (PCC) more likely to progress from mild cognitive impairment (MCI) to AD (Hunt et al. 2007, Fouquet et al. 2009, Zhang et al. 2012) as well as differentiating between multiple dementia types as will be discussed below.

The hippocampus is a key region for memory and learning and is particularly susceptible to degradation in AD. Thus it should be mentioned that there is some disagreement within the field as to whether the hippocampus displays hypometabolism in AD. Some have argued that the hippocampus does not show reductions (Minoshima et al. 1997, Ishii et al. 1998) while others have argued for quite striking reductions (Nestor et al. 2003, Mosconi et al. 2005, Maldjian et al. 2012). This issue has largely arisen depending on the type of analysis used to examine FDG-PET images. Studies that have used a voxelwise approach are unlikely to show hippocampal metabolism, however this method is prone to error, partly due to issues with mapping of brain scans to templates in disease states. In contrast, use of a region-of-interest analysis with correction for spatial normalisation yields a more accurate picture and confirms the hippocampal hypometabolism (Maldjian et al. 2012).

1.10.1 Hypometabolism exists as a distinct phenotype from atrophy

One of the common criticisms against the apparent reductions in glucose metabolism within the AD brain is that it can easily be explained by the extent of cell death and atrophy of the areas under investigation. Naturally if there is less brain tissue within a given region (replaced by enlarged CSF space) this will decrease the amount of glucose taken up and cause the tissue to appear hypometabolic. However numerous studies have directly addressed this issue. The most common technique to account for atrophy is for an MRI scan to be taken in addition to the PET imaging. This allows correction for the volume effect and would in turn decrease the apparent level of hypometabolism. This was seen to be precisely the case in AD with an apparent increase in CGU of between 19 and 50% (Meltzer et al. 1996). However, of importance is that increases of between 16 and 38% were also noted within the control group. Further, significant hypometabolism was present both before and after correction in the Alzheimer's group in the frontal, posterior temporal, and parietal regions when compared to the controls. This is supported by (Ibáñez et al. 1998) who again showed that after partial volume correction, significant hypometabolism is present within AD patients and this trend is even noted in preclinical dementia (Kljajevic et al. 2014)

The extent of hypometabolism also exceeds the extent of atrophy in several brain regions, particularly within the PCC and precuneus (Chételat et al. 2008) and in those with familial AD, hypometabolism is apparent before structural alterations begin (Mosconi et al. 2006). In addition to demonstrating that hypometabolism exists as a distinct phenotype to atrophy, it also shows that the magnitude of hypometabolism can be predictive of disease risk and severity.

1.10.2 Cerebral hypometabolism can be used to differentiate between multiple dementia forms

A difficulty in the diagnosis of AD is that memory deficits are key symptoms of a number of other dementias such as frontotemporal dementia, dementia with Lewy Bodies, and vascular dementia. This means that even in the case that a successful disease-modifying drug therapy has been developed for AD, the lack of diagnostic clarity would present a major challenge in the assessment of drug efficacy. As discussed previously, PET imaging displays very distinct patterns of hypometabolism within the brains of those with AD. This begins first in the cingulate cortex, progressing to the medial temporal lobe before moving the frontal corticies, and these patterns could potentially be used to differentiate AD from other neurological conditions, as discussed below.

Vascular dementia is considered to be second most common form of dementia behind AD. While the vascular form of AD also shows hypometabolism within limbic areas similar to AD, it is differentiated by large patterns of reduced metabolism within the frontal cortex. Using both ^{18}FDG-PET (Kerrouche et al. 2006) and oxygen-15 labelled compounds (another method of assessing cerebral glucose metabolism) (Nagata et al. 2000), it has been possible to reliably differentiate between the two diseases.

With regard to frontotemporal dementia, it is characterised by reduced metabolism within the frontal lobes, particularly in the early stages of the disease. As the disease progresses, this hypometabolism spreads throughout the brain with the caudate and thalamic areas showing large reductions with some smaller reductions in the limbic areas (Ishii et al. 1998, Diehl et al. 2004, Diehl-Schmid et al. 2007). Given these differences compared to AD, several studies have shown the PET scanning is able to either improve diagnosis or differentiate

frontotemporal dementia from AD (Higdon et al. 2004, Foster et al. 2007, Rabinovici et al. 2011).

Finally, in dementia with Lewy Bodies there is again somewhat similar patterns of hypometabolism when compared to AD with reductions present within the limbic areas (Ishii et al. 1998), however, the disease is differentiated by large reductions in the primary visual cortex and also within the temporo-pariteo-occipital associations cortices (Imamura et al. 1997, Imamura et al. 1999, Higuchi et al. 2000, Fujishiro et al. 2012). When compared to AD, these distinct patterns of hypometabolism make it easy to differentiate between the two diseases (Higuchi et al. 2000, Minoshima et al. 2001). Taking all these factors into account, a multicenter study examined 18F-FDG PET for the diagnosis of AD vs. normal aging, MCI, and other dementia forms and showed that using standardized disease-specific PET patterns it was possible to correctly identify 95% AD of cases (Mosconi et al. 2008).

1.10.3 Biochemical impact of hypometabolism

In terms of the consequences of reduced glucose metabolism, the major theory has been that it results in an increase in oxidative stress. Occurring parallel to the energy producing glycolytic pathway in neurons is the pentose phosphate pathway (PPP) (Bolaños et al. 2010), that uses glucose to generate NADPH (Stincone et al. 2015). In turn, NADPH is essential for redox reduction action and its depletion can lead to oxidative damage, a common feature in both mild cognitive impairment (Butterfield et al. 2006) and AD (Nunomura et al. 2001, Bhat et al. 2015). This can also contribute to a cycle of damage and reduced uptake, with oxidative damage of mitochondria noted in AD (Hirai et al. 2001), and similar to hypometabolism preceding clinical symptoms of AD, this oxidative damage also seems to precede typical AD pathology (Nunomura et al. 2001). It is this pathway that has been suggested as a possible mechanism by which hypometabolism could be a causative factor in the development of AD. These changes will be discussed with particular regard to the mouse models below.

1.10.4 Alzheimer's Disease Pathogenesis Summary

While amyloid and tau are both pathological features of Alzheimer's disease, the question as to whether they are the cause of AD or consequence of its development is still unclear. Despite this, much early research has been centered on the fact that a series of familial mutations which only cause a small proportion of AD cases.. In turn, this has lead to the generation of numerous mouse models based on these genetic mutations. Several drug therapies have demonstrated very promising results in these models, often showing near complete reduction in pathology. However, these same drugs invariably go on to fail in clinical trials (Green et al. 2009, Quinn et al. 2010, Hung et al. 2017). Given that many of the models are solely amyloid based and do not show extensive neuronal loss (Games 1995, Oakley 2006), this is perhaps not so surprising. Despite the association between hypometabolism and disease progression in human AD, the progression of mouse hypometabolism has received relatively little study. Thus, if it is not present in the mouse models this highlights that there is a substantial deviation from the human condition and could go some way towards explaining the failure of promising drug candidates.

This forms the crux of the current review, which aims to draw together that current studies that have investigated CGU in the various mouse AD models and comment on the current standing of the field.

1.11 Mouse models of AD

All the mouse models relevant to the investigation of cerebral glucose uptake were generated though inserting the human genes associated with increased AD risk into mice. Several models such as the 3xTg and 5xFAD also incorporate a mutation in either of the Presenilin (*PSEN*-1 or *PSEN*-2) genes alongside the *APP* mutations. Originally discovered in a number of families who displayed familial AD (but who did not appear to have mutations in *APP*), *PSEN-1 and -2* mutations alter the processing of amyloid beta, shifting the expression profile towards the more aggregate prone Aβ42 oligomer (Kaneko et al. 2007), in turn increasing the risk of the disease. In addition to these mutations, some of the more recently generated strains of the AD mice also include tau mutations, specifically in the *MAPT* (Microtubulue-Associated Protein Tau) gene the *P301L* mutation, which features a proline to leucine substitution at position 301 (Oddo et al. 2003). This leads to these mice developing tau tangles similar to the human condition. However, these mice still do not display neuronal loss, similar to the vast majority of the plaque only models.

There are a multitude of models available, so this review will limit discussion of the mouse models to those that have been examined with respect to glucose metabolism within the brain. Thus, this section aims to give a perspective to when the pathological and memory impairments are occurring in relation to their reported hypometabolism, (all references to differences, for example hypometabolism noted in the hippocampus, refer to age matched transgene controls).

1.11.1 Cerebral glucose uptake phenotype of AD mouse models

There are two methods commonly used to assess glucose uptake within mouse models, autoradiography and positron emission tomography (PET), both utilising radiolabelled glucose. This section will provide a brief overview of each method and the relative benefits are then discussed

For autoradiography (AR), mice are generally given an intraperitoneal injection and following an uptake period (usually 45 minutes) the mice are killed and brains sectioned before being exposed to a radiographic film. While this does not allow tracking the same animal across disease progression, it does

allow for the animal to be freely roaming during the uptake period and offer excellent spatial resolution. In contrast, during PET, mice are first anesthetized to prevent movement, before being given an intravenous injection of radiolabelled glucose. This allows a 3D reconstruction of the brain with regards to glucose uptake and allows tracking of the same animal during the disease process, particularly relevant in studies examining a drug effect.

Below is a table summary of the studies that have examined cerebral glucose uptake in mouse models of AD. While not discussing each individual study in detail, these serve to highlight the disparity within the literature. Two regions were chosen, the hippocampus and retrosplenial cortex, as they are affected early in the progression of AD-like pathology, particularly with regard to CGU. The whole brain average (the mean of all cerebral glucose uptake) was also chosen given that this reflects the overall state of the brain's metabolic status. Of note is that the whole brain average cannot be determined accurately using autoradiography, thus an attempt to examine and summarise the main findings of those studies was presented.

1	(Dodart et al. 1999)	**2**	(Reiman et al. 2000)	**3**	(Valla et al. 2008)
4	(Sadowski et al. 2004)	**5**	(Valla et al. 2006)	**6**	(Dubois et al. 2010)
7	(Lebenberg et al. 2010)	**8**	(Takkinen et al. 2016)	**9**	(Nicholson et al. 2010)
10	(Niwa et al. 2002)	**11**	(Coleman et al. 2017)	**12**	(de Cristóbal et al. 2014)
13	(Poisnel et al. 2012)	**14**	(Rapic et al. 2013)	**15**	(Wang et al. 2012)
16	(Wang et al. 2013)	**17**	(Li et al. 2016)	**18**	(Liu et al. 2017)
19	(Kuntner et al. 2009)	**20**	(Luo et al. 2012)	**21**	(Köfalvi et al. 2016)
22	(Martín-Moreno et al. 2012)	**23**	(Ye et al. 2016)	**24**	(Ding et al. 2013)
25	(Sancheti et al. 2013)	**26**	(Rojas et al. 2013)	**27**	(Macdonald et al. 2014)
28	(Xiao et al. 2015)	**29**	(Waldron et al. 2015)	**30**	(Deleye et al. 2016)
31	(Waldron et al. 2017)	**32**	(Brendel et al. 2016)		

Table 1-2 List of studies that have examined cerebral glucose uptake in AD transgenic mouse lines

Green = No difference from wild-type

Blue = Hypometabolism

Red = Hypermetabolism

Orange = Unclear, some sub-regions elevated, some decreased

Whole Brain Average

	Study	Strain	Age 0 > 3	4 > 7	8 > 11	12 > 15	16 > 19	20 > 23
Autoradiography	1	PDAPP						
	2	PDAPP						
	3	PDAPP						
	4	PSAPP						
	5	PSAPP						
	6	PSAPP						
	7	PSAPP						
	8	PSAPP						
	9	3xTg						
	10	Tg2576						
	11	Tg2576						
	12	TASTPM						
PET Scanning	13	PSAPP						
	14	PSAPP						
	15	PSAPP						
	16	PSAPP						
	8	PSAPP						
	17	PSAPP						
	18	PSAPP						
	19	Tg2576						
	20	Tg2576						
	21	Tg2576						
	22	Tg2576						
	11	Tg2576						
	23	3xTg						
	24	3xTg						
	25	3xTg						
	26	5xFAD						
	27	5xFAD						
	28	5xFAD						
	12	TASTPM						
	29	TASTPM						
	30	TASTPM						
	31	TASTPM						
	32	PS2APP						

Table 1-3 Comparison of whole brain average cerebral glucose uptake phenotype across age and strain of AD transgenic mice

Retrosplenial Cortex

	Study	Strain	Age					
			0 > 3	4 > 7	8 > 11	12 > 15	16 > 19	20 > 23
Autoradiography	1	PDAPP						
	2	PDAPP						
	3	PDAPP						
	4	PSAPP						
	5	PSAPP						
	6	PSAPP						
	7	PSAPP						
	8	PSAPP						
	9	3xTg						
	10	Tg2576						
	11	Tg2576						
	12	TASTPM						
PET Scanning	13	PSAPP						
	14	PSAPP						
	15	PSAPP						
	16	PSAPP						
	8	PSAPP						
	17	PSAPP						
	18	PSAPP						
	19	Tg2576						
	20	Tg2576						
	21	Tg2576						
	22	Tg2576						
	11	Tg2576						
	23	3xTg						
	24	3xTg						
	25	3xTg						
	26	5xFAD						
	27	5xFAD						
	28	5xFAD						
	12	TASTPM						
	29	TASTPM						
	30	TASTPM						
	31	TASTPM						
	32	PS2APP						

Table 1-4 Comparison of cerebral glucose uptake phenotype in the retrosplenial cortex across age and strain of AD transgenic mice

Hippocampus

	Study	Strain	Age					
			0 > 3	4 > 7	8 > 11	12 > 15	16 > 19	20 > 23
Autoradiography	1	PDAPP						
	2	PDAPP						
	3	PDAPP						
	4	PSAPP						
	5	PSAPP						
	6	PSAPP						
	7	PSAPP						
	8	PSAPP						
	9	3xTg						
	10	Tg2576						
	11	Tg2576						
	12	TASTPM						
PET Scanning	13	PSAPP						
	14	PSAPP						
	15	PSAPP						
	16	PSAPP						
	8	PSAPP						
	17	PSAPP						
	18	PSAPP						
	19	Tg2576						
	20	Tg2576						
	21	Tg2576						
	22	Tg2576						
	11	Tg2576						
	23	3xTg						
	24	3xTg						
	25	3xTg						
	26	5xFAD						
	27	5xFAD						
	28	5xFAD						
	12	TASTPM						
	29	TASTPM						
	30	TASTPM						
	31	TASTPM						
	32	PS2APP						

Table 1-5 Comparison of cerebral glucose uptake phenotype in the hippocampus across age and strain of AD transgenic mice

1.11.2 Current consensus

Despite the overall lack of agreement across studies, the most promising in terms of matching the human model is the consistency of hypometabolism observed in the RSC/Cingulate cortex, a key early region in the pathogenesis of human AD. While none of the PET scanning studies discussed here examined this region, as it is often below the spatial resolution of the scanners, it was assessed in the majority of the autoradiographic studies. With the exception of (Sadowski et al. 2004) and(Valla et al. 2006), where the RSC itself was hypometabolic while the other regions of the cingulate where unchanged, all of the studies noted a reduction in glucose utilization and is further strengthened as the effect is noted across multiple strains.

When considering the other key brain regions however, the evidence is far less clear. A reduction in whole brain metabolism is another characteristic feature of AD, particularly as the disease progresses. However, even in the older animals examined here, only a small portion demonstrate this phenotype. Perhaps the biggest factor to consider is the phenotype of the hippocampus, another key and early affected region in human AD. Across all of the examined studies, the hippocampus shows hypometabolism in only a few studies and even hypermetabolism in others.

This leads to an interesting hypothesis regarding the mouse models as a whole. While the animals display the characteristic early glucose dysregulation in the RSC/cingulate, they do not the show the later aspects of the disease, such as the reduction in hippocampal and whole brain metabolism. This could suggest that amyloid is beginning to have its deleterious effects on the brain and simply does not have enough time to fully exert its effects in the two year life span of the mouse as opposed to the decades of action in the human condition. Another possibility is that the mouse biology is more capable of dealing with the insult caused by the amyloid accumulation. In either case, considering the implications for IRAP again, clarification of the mouse phenotype is crucial. As glucose uptake likely plays a key role in mediating the effects of IRAP-I action, particularly the enhancement of cognition, the success of the inhibitors could depend on the animals demonstrating a hypometabolic phenotype. In order to

do this I believe there are several methodological issues to address in order to accurately characterize the mice.

1.11.3 Current issues in phenotyping the CGU of AD mice

1.11.3.1 Resolution of FDG-PET

Many of the regions investigated in the previously mentioned studies are relatively small, often bordering on or well below the spatial resolution of most microPET scanners. Valla and colleagues (2002) demonstrated that a full-width-half-max (FWHM, the resolution of the scanner) of 0.5mm or less is required to accurately detect hypometabolism in small regions such as the posterior cingulate cortex. Considering all of the scanners in the studies mentioned previously had a FWHM of at best 1.3mm it is reasonable to question how accurately spatially small regions can be measured.

In direct examination of the ability of microPET to detect differences between groups, no differences were found in the Tg2576 mice(Kuntner et al. 2009), however, this involved a limited sample size and single age of examination. A previous study reported that there is a good agreement between cerebral glucose uptake (CGU) measured by both PET and autoradiography (Toyama et al. 2004). The authors acknowledge that this is only true when relatively large regions of interest are used and thus still calls into question the reliability of previous studies.

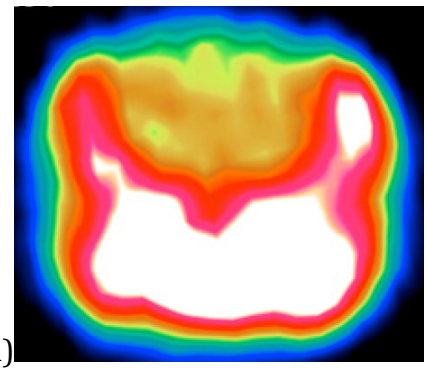
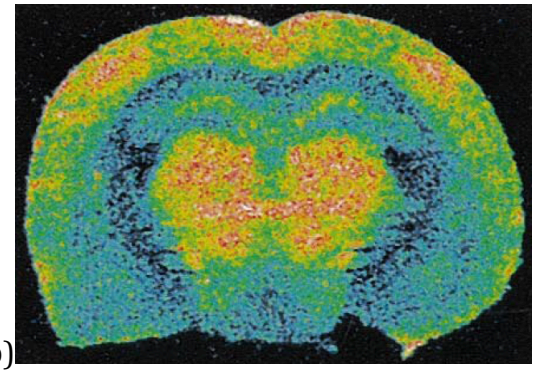
a)  b) 

Figure 1-7 Comparison of spatial resolution of CGU between a) FDG-PET and b) autoradiography

As seen in a) there is clear bleeding of the signal in adjacent brain regions, whereas in b) individual structures such as the hippocampus can be visualized reliably. Images taken from a) (Wang et al. 2012) b) (Dodart et al. 1999)

1.11.3.2 Effect of anaesthesia

While PET assessment in humans is performed with the subject awake, microPET necessitates anesthesia in order to immobilise the animal. While several studies do allow for glucose uptake while the animal is free roaming in the cage before inducing for the scan, others administer the glucose tracer under anesthesia before allowing a conscious uptake period and some perform the entire uptake and PET scan under anesthesia.

It is well documented that overall CGU is seen to decrease under several types of anesthesia in a number of species (Shapiro et al. 1978, Toyama et al. 2004, Lee et al. 2010).. Most importantly however, is that the type and level of anesthesia can show regional variability, for example propofol affects cortical regions at lower doses and subcortical areas such as the hippocampus at higher doses (Sun et al. 2008), while isoflurane consistently results in lower uptake compared to ketamine/xylazine (Toyama et al. 2004). Beyond this, both isoflurane and ketamine/xylazine are noted to cause acute hyperglycaemia following administration (Saha et al. 2008).

1.11.3.3 Sex of animals

In human studies females have consistently higher cerebral glucose uptake compared to males (Baxter Jr et al. 1987, Willis et al. 2002) and also

show regional variability across the estrous cycle within key areas such as the thalamus, retrosplenial cortex and cingulate (Reiman et al. 1996). Several of the mouse studies have used either mixed or exclusively female cohorts with no mention of estrous cycle matching in either case.

1.11.3.4 Fasting period

The rate at which the glucose tracer is taken up is also dependent on the level of circulating endogenous glucose that could compete for entry into the cell and thus prevent some tracer from being absorbed (Fueger et al. 2006). This is further compounded by the observation that some AD mouse strains have lower blood glucose levels than their transgene negative controls (Deleye et al. 2016, Takkinen et al. 2016), thus apparent hypermetabolism may simply be the result of there being less competition for tracer uptake. A caveat to note here is that these experiments were performed measuring peripheral glucose uptake and have not been replicated in the CNS, nonetheless, given the variability of animal data, it is an important point to note.

1.11.3.5 Temperature

Body temperature can also affect the distribution of glucose tracer around the body of the animal with colder temperatures causing an increase in the distribution to brown fat (Fueger et al. 2006). This effect is especially pronounced when animals are anaesthetised, with body temperature falling by 25%. There is a roughly 4-fold difference in the levels of brain uptake between animals that are not fasted or heated and those that are (Fueger et al. 2006). As the metabolic rate of mice is close to 7-fold higher than humans (Kleiber 1961), small differences in animal status can lead to large shifts in glucose distribution.

1.11.3.6 Choice of normalisation region

Alzheimer's disease is associated with reduced cerebral perfusion making it vital to normalise glucose uptake against a reference region to account for variability in the amount of tracer reaching cerebral tissue. An ideal reference region should be stable between groups, free from pathology and easy to identify.

One of the most common regions chosen is the cerebellum, which, while being relatively spared from AD pathology in the early to mid-stages of the disease, has been noted be affected in the late stages (Yamaguchi et al. 1989) at which point CGU is also affected (Ishii et al. 1997). Despite reportedly not displaying plaques, the cerebellum is still reported to express APP (Nicholson et al. 2010) and variable uptake has been noted within the cerebellum in the 5xFAD model (Macdonald et al. 2014). Thus, while appropriate in the early stages of disease progression the cerebellum may be become compromised in the later stages.

Normalisations against the whole brain average are also often used, which are useful due to the ease of identification and increased signal-to noise ratio. However, in cases of brain-wide reduction is CGU (such as AD), normalisation against the whole brain can remove group differences and create hypermetabolic artefacts (Yakushev et al. 2008, Dukart et al. 2010, Küntzelmann et al. 2013).

1.11.3.7 Future directions for glucose metabolism research in AD mice

As has been highlighted, there is substantial variability in the reports of glucose metabolism within the brains of AD mice with differences not only between models but also between studies examining the same strain. In order to phenotype the AD mouse accurately there are a number of factors that should be taken into account.

1. More studies must be performed within the existing models to add to the current data and hopefully reduce variability. As it stands, two studies can provide almost completely conflicting results making meaningful interpretation difficult.
2. Close attention should be paid to the sex of the animal cohorts used with efforts made to use exclusively one sex and in the case of female use, that estrous cycle matching is performed.
3. Animals should be fasted for 8 - 16 hours prior to uptake to ensure that baseline blood glucose levels are comparable both within and between groups.

4. Particularly in cases where animals are anaesthetised, mice should be placed on heating pads and body temperature monitored
5. In the case of PET studies, glucose administration and uptake should occur while the animal is conscious in order to more accurately reflect the metabolic activity of the brain.
6. Autoradiographic studies should take precedence over those performed with PET scanning as the spatial resolution afforded by autoradiography is vital to examining relatively small regions such as the hippocampus and retrosplenial cortex, which are central regions in AD progression at large, but also particularly in the progression of hypometabolism.

Making these changes should improve the quality of data regarding glucose metabolism in the AD mouse brain and make comparisons to the human condition far easier. In turn this will provide more evidence as to the suitability of the mice to function as a model for the human condition.

1.11.4 Molecular mechanisms of hypometabolism

As discussed, there are several issues that make it difficult to specifically phenotype the AD mouse model. While not a direct examination of CGU, investigation of the molecular metabolic dynamics in the AD mouse brain can provide indirect evidence to support or explain the results from autoradiographic and PET studies. From human studies there is evidence to suggest that portions of the metabolic pathway are deficient in AD, glucose-6-phosphate dehydrogenase activity is reduced in the hippocampus for example, (Bigl et al. 1999), while a number of genes associated with energy metabolism are down regulated in the cingulate (Loring et al. 2001). Thus an investigation into whether some of these traits are present in the mouse models will provide a more detailed understanding of how closely the mice match humans.

1.11.4.1 Insulin

There has been much discussion within the human literature of the role that insulin plays in the development of AD, particularly following the evidence of glucose hypometabolism within the brain, with AD sometimes being referred to as 'Type III' diabetes. There is certainly much evidence to support the role of insulin in cognition; insulin mRNA and the receptor are expressed in key areas

such as the hippocampus (Devaskar et al. 1994), administration of insulin can promote memory enhancement (Park et al. 2000, Stern et al. 2014) and insulin signalling dysfunction has been associated with cognitive impairment (Convit et al. 2003). For a comprehensive review of the interaction of insulin and cognition in the animal models readers are directed here (Calvo-Ochoa et al. 2015).

There are two main proposed mechanisms by which insulin could play a role in the development of AD. The first suggests that insulin resistance developing within the brain leads to a reduction in the activity of downstream signalling activity, reducing pro-survival mechanisms (Steen et al. 2005), while also increasing the activity of GSK3β leading to increased tau hyperphosphorylation (Steen et al. 2005). The second involves the interaction with insulin degrading enzyme (IDE), which has been indicated as the main amyloid degrading enzyme, thus the presence of excessive insulin can reduce the degradation of Aβ (1-40) and Aβ (1-42) resulting in increased plaque accumulation (Pérez et al. 2000).

In regard to the mouse models however, the evidence is less clear. Interference with the insulin signaling pathway leads to worsened pathology and cognition. Streptozotocin induced diabetes results in increased tau phosphorylation (Clodfelder-Miller et al. 2006, Ke et al. 2009) and impaired performance on the Morris water maze (Sonn et al. 2015). Yet, there is little evidence in the animal models to suggest the reverse. Thus, in regards to glucose metabolism within the brain, it appears unlikely that insulin plays a role in the observed phenotype in the mice.

1.11.4.2 Glycolytic enzymes

In line with this, 24 month old Tg2576 transgenic mice display a reduction in phosphofructokinase mRNA as well as protein level and activity in the cerebral cortex (Bigl et al. 2003). Considering the 3xTg model, the level of pyruvate dehydrogenase was reduced by approximately 30% across all examined ages (3,6,9 and 12 months old). A comprehensive mitochondrial proteome analysis from the cortices of six-month-old mice demonstrated dysregulation in a variety of genes (Chou et al. 2011), mirroring results seen from human patients (Melanson et al. 2006).

1.11.4.3 Mitochondrial enzymes

Several lines of evidence have suggested that reasons for the reduction in neuronal metabolism are due to direct effects of amyloid oligomers on mitochondria, reducing not only their number but also efficiency. In turn, this reduces the metabolic capacity of the cell and leads to a reduction in glucose uptake and apparent hypometabolism, especially in hippocampal neurons (Keller et al. 1997, Mark et al. 1997, Prapong et al. 2001, Uemura et al. 2001, Prapong et al. 2002). In turn, this leads to a reduction in TCA cycle metabolites and ATP production (Zhang et al. 2015, Andersen et al. 2016). These effects appear to be due to an increased oxidative stress placed upon the mitochondria with amyloid increasing lipid peroxidation (Du et al. 2010, Yao et al. 2011) and antioxidants able to rescue glucose uptake (Keller et al. 1997).

Several oxidative stress makers are noted to be upregulated in the brains of transgenic AD mouse models. Protein and lipid stress markers are associated strongly with fibrillar β-amyloid (Matsuoka et al. 2001). Two general oxidative damage markers, superoxide dismutase (SOD) and hemoxygenase-1 (HO-1), are upregulated particularly around dystrophic neurites (Pappolla et al. 1998). Neurites close to plaques have fewer mitochondria, an effect seen in both humans (Pérez-Gracia et al. 2008) and also the Psd9 mouse (Xie et al. 2013). As well as reducing in number, mitochondrial function is also affected with soluble amyloid interacting directly with the mitochondrial membrane, the site of many respiratory transporters and enzymes (Manczak et al. 2006, Du et al. 2010). Complex IV (cytochrome c oxidase), the enzyme responsible for ATP production, seems to be particularly vulnerable with reductions in content noted both *in vitro* (Manczak et al. 2006) and *in vivo* (Yao et al. 2009, Du et al. 2010, Pedrós et al. 2014, Andersen et al. 2016). Interestingly, Du and colleagues found that soluble amyloid is more strongly associated with synaptic mitochondria compared to other areas of the neuron, suggesting a direct effect of neuronal transmission rather than the overall metabolism of the cell.

1.11.4.4 Glucose transporters

As mentioned previously, there are three main glucose transporters present within the brain. GLUT1 is localised in astrocytes and is the main

glucose transporter responsible for transporting glucose into the brain (Klepper et al. 2002). GLUT3 is largely present in neurons and contributes to maintain basal glucose uptake (McCall et al. 1994). GLUT4 is also present on neurons and functions as an inducible glucose transporter, allowing for increased glucose uptake in response to neuronal activation (Fernando et al. 2008).

In humans AD, a reduction of both GLUT1 (Horwood et al. 1994, Mooradian et al. 1997) and GLUT3 (Simpson et al. 1994, Harr et al. 1995) has been noted, particularly within the hippocampal formation. In mice, GLUT1 reductions have been observed in the 3xTg model (Ding et al. 2013) and also specifically within the hippocampal formation of the APP/PS1 mouse (Hooijmans et al. 2007). In terms of the neuronal glucose transporters, a reduction in GLUT4 is noted within the brains of 7-month and 13-month old 3xTg mice (Sancheti et al. 2013) while GLUT3 was only observed in the 13-month old mice. The results of the 3xTg model are particularly interesting, where Ding and colleagues (2013) noted that while there was a decrease in glucose uptake with age this was not significantly different from controls animals at any age. Thus, despite the reduction in transporter levels, the mice were utilising an equivalent amount of tracer, suggesting a possible compensation mechanism is occurring. Indeed, mice haploinnsufficient for GLUT3 had similar levels of glucose uptake as wild-type littermates (Stuart et al. 2011), suggesting that reductions in the passive glucose transporters may not affect overall brain glucose metabolism.

1.11.4.5 Supplementary energy sources

As the there is a clear reduction in glucose uptake within the AD brain an obvious solution is to provide an alternative energy source to sustain cellular processes. Ketogenic diets are already used in the treatment of epilepsy disorders in which there is GLUT1 deficiency and recent evidence has suggested they may form a possible therapeutic option in AD. During periods of fasting or during a low calorie diet, fatty acids are released from stores where they are metabolised within the liver to form ketone bodies, which in turn can be used by neurons as an alternative energy source.

The brain is well suited to using ketone bodies as an alternative fuel source with up to 60% of its energy supplied by these during periods of starvation (Owen et al. 1967) and also in disease states such as GLUT1 deficiency (Klepper et al. 2004) as well as numerous others, for review see (Gasior et al. 2006). There is already evidence that inducing ketosis in human Alzheimer's can have beneficial effects, dietary supplementation with medium chain triglycerides improved cognitive performance with acute delivery (Reger et al. 2004) and after 90 days of continued administration (Henderson et al. 2009).

Thus, if ketogenic diets can ameliorate pathology and symptoms of AD, this could imply that the cells are in a state of metabolic distress and are relying on an alternative energy source, which in turn provides indirect evidence that hypometabolism is present.

1.11.4.6 Effect on AD Mouse models

Ketogenic diets appear to have a positive effect on the histological and biochemical pathology associated with AD in the mouse models. Eight month old female 3xTg mice fed a ketogenic diet for 7 weeks showed a reduction in proteins associated with damage such as HSP60 and MnSOD as well as an increase in metabolic enzymes such as α-ketoglutarate (Yao et al. 2011). In the PDAPP model, three month old female mice fed a ketogenic diet for 43 days showed reductions of roughly 25% in brain Aβ 40 and 42 protein levels (Van der Auwera et al. 2005). In terms of cognitive outcomes, eight month old 3xTg male mice were placed on ketogenic diet for eight months and displayed improved memory performance as well as reduced amyloid plaque and tau pathology (Kashiwaya et al. 2013). While not a ketogenic diet, a study by Isopi and colleagues demonstrated that supplementation with pyruvate improved performance on the Morris water maze task and reduced oxidative damage (Isopi et al. 2015).

However, these studies are contrasted by those showing no benefit in response to a ketogenic diet. APP_{SWE} x $PS1_{P246L}$ mice did show an improvement in motor performance, however no change to soluble Aβ was noted. An important note to this study is that the animals were only placed on the diet for

1 month and culled at 3 months of age. At this age soluble Aβ levels and plaques are often absent or present in very small amounts (Gordon et al. 2001, Murphy et al. 2007). A second study investigated the APP_{SWE} x $PS1_{M146L}$ mice as well as a Tau model, the Tg4510. The ketogenic diet improved motor performance in all groups, however, it was unable to improve cognition in a number of paradigms including fear condition, Y-maze task and the radial arm water maze Brownlow et al. 2013).

- Another issue that makes interpretation of these results difficult is the varied means by which ketogenic was induced in each study. Diets included;
- High protein / low carbohydrate (Aso et al. 2013)
- High fat / low carbohydrate (Van der Auwera et al. 2005, Beckett et al. 2013, Brownlow et al. 2013)
- Addition of 2-Deoxy-Glucose to regular rat chow (Yao et al. 2011)

As mentioned earlier, medium chain triglycerides were used to obtain the positive cognitive results seen in human AD, a diet not replicated by any of the animals studies here. Further, the low carbohydrate diets could have unpredicted side effects that might explain some of the variability between the studies, particularly in regards to cognition.

1.11.4.7 Summary of molecular mechanisms

Overall it appears that the mouse models of AD display a variety of metabolic deficits at the molecular level including decreased transporter and respiratory enzyme levels. The largely positive effects of the ketogenic diet suggests that supplementation with an alternative energy source could be alleviating metabolic distress within cells. As mentioned above, it is difficult to determine cause/effect from these results. Whether specific reductions in enzymes are the cause of cellular dysfunction or merely due to an overall dysregulation induced by other factors such as amyloid or tau remains unclear. A more detailed examination of the expression of key metabolic proteins is needed, in particular, a time course of the reductions in specific brain regions.

While there appears to be a convincing story for the role of amyloid oligomers in disrupting the function of mitochondria and leading to a reduction in neuronal metabolism, there are some caveats to consider. Most notable are the separate early patterns of amyloid deposition and hypometabolism, the former beginning first in the association cortices and the latter beginning in the medial temporal lobe. If amyloid were the cause of the hypometabolism it would be expected that the two would share a temporal/spatial pattern. Thus while it appears that amyloid may be cytotoxic in its own right there are likely a variety of other pathological processes taking place independently to reduce glucose metabolism.

1.11.5 Summary of AD mouse hypometabolism

In summary, whether the mouse models of AD accurately represent the human disease in regards to glucose metabolism is still unclear. The most consistent feature seen across multiple models is the reduction in glucose uptake in the posterior cingulate cortex, an early region affected in human AD. Unfortunately, this is not replicated in the hippocampus, another key area in the pathological progression of the disease. Given the repeated failure of many promising drug candidates in human trials, it is vital to fully characterise the animals used to model the disease. This is particularly relevant in the case of the IRAP-Is as one of the potential mechanisms involves modulating glucose uptake into neurons. While the animals are only models of the human condition, whether they replicate the hypometabolism of the human condition is key to define in assessing the IRAP-Is.

1.12 Summary and Directions

While inhibition of IRAP by both peptide and synthetic inhibitors has been demonstrated to enhance memory across a variety of paradigms, the mechanism remains unclear. This is made more difficult given the broad distribution of IRAP and the numerous suggested functions it performs. Further there is also interest as to the potential efficacy of the IRAP inhibitors in diseases such as AD. This forms the primary motivation of the current thesis, which is to characterize the models in which the IRAP-Is are tested, in order to gain further insight as to their possible modes of action.

To date, IRAP inhibitors have exclusively been administered directly into the lateral ventricles of the brain via indwelling cannulas in order to study their effects on cognition. However, whether this surgery itself causes a deficit model is unclear. This question formed the work of Chapter 2 where I characterized the behavioral and cellular effects of i.c.v surgery model so that more definitive assumptions can be made about the potential action of the IRAP-Is. Following from this work, in Chapter 3 I developed an *in vitro* assay to serve as a screen for novel cognitive enhancing IRAP inhibitors. This also allowed an investigation of the likely mechanisms of action of cognitive enhancement, increased glucose uptake and altered MMP expression. As the precise phenotype of the AD mouse is currently unclear, particularly in regard to glucose metabolism, in Chapter 4 I have investigated the 5xFAD mouse model with particular regard to those features. Here I also performed preliminary work to investigate the potential of the IRAP-Is as therapeutics in the disease. The summary and conclusions of this work is presented in Chapter 5 and pilot data presented in Chapter 6.

Chapter 2

Characterisation of the behavioural consequences of intracerebroventricular cannulation

2.1 Introduction

Inhibitors of IRAP are well documented for their ability to enhance memory and learning across a wide variety of behavioral paradigms including passive avoidance tasks (Braszko et al. 1988, Wright et al. 1993) and spatial tasks such as the Morris water maze (Wright et al. 1999) and Barnes circular maze (Lee et al. 2004). IRAP-Is are also capable of rescuing experimentally induced deficits, such as those caused by chronic alcohol exposure (Wisniewski et al. 1993) and global ischemia (Wright et al. 1996). Despite these promising results, the mechanism behind this enhancement remains unknown. Given the multi-factorial role that IRAP likely plays, a better understanding of the paradigms in which the inhibitors are tested could lead to valuable clues as to the potential mechanism of action governing IRAP-I mediated cognitive enhancement.

Currently, testing of the IRAP-Is is still in early preclinical stages and as such, the compounds are administered directly into the lateral ventricles of the brain through a chronic indwelling cannula. This method of delivery is routinely used to deliver pharmacological agents directly into the brain, both to ensure targeted action and to avoid difficulties with permeability across the blood brain barrier (Albiston et al. 2008). Often the claims resulting from these studies are that the specific cognitive enhancing agents (nootropics) are able to enhance 'normal' memory function. Work investigating a rescue of a cognitive deficit generally involves the infusion of a compound to induce neuronal dysfunction or cell death, such as scopolamine (Parsons et al. 1992) or kainic acid (Shetty et al. 1999).

However, given the invasive nature of intracranial cannulations, it is possible that the surgical procedure itself could cause significant damage to the brain and contribute to, or cause, an impaired state. Indeed, common surgical procedures similar to i.c.v cannulations have been demonstrated to impair memory function. Implantation of a metal cannula into the striatum (Frumberg et al. 2007) and insertion of an electrode into the subthalamic nucleus (Hirshler et al. 2010) have both been shown to impair recognition memory. Cognitive deficits resulting from brain injury are also well documented in the literature in

both humans (McDowell et al. 1997, Palacios et al. 2013) and animal models (Sweet et al. 2014). Cerebral inflammation has been associated with memory deficits (Wellmer et al. 2000) and in particular both TNF-α and IL-6 are upregulated following traumatic brain injury and have been independently associated with cognitive impairments (Butler et al. 2004, Sparkman et al. 2006, Ren et al. 2011). Relevant to the current work is that IRAP is known to play a role in various pathological processes including cancer (Shibata et al. 2005) and stroke (Pham et al. 2012) and there have been reports of IRAP expression within astrocytes following cortical stab injury (*Fernando*, unpublished*).*

These impairments become especially relevant considering that the cellular and molecular mechanisms that govern 'normal' memory enhancement and those that are involved in rescuing a memory deficit may be completely distinct. This in turn introduces a confounding factor when attempting to elucidate the potential mechanisms of the nootropic agents. It is conceivable that the cognitive enhancing effects of IRAP could be due to a suppression of inflammation for example. Thus, in order to elucidate the mechanism of action that is driving the enhancement of cognition in response to IRAP-Is, an understanding of the paradigm in which they are being tested is required.

The present study aimed to investigate the behavioural and cellular effects of an indwelling cannula implanted unilaterally into the lateral ventricle, a common route of nootropic compound delivery and used in the investigation of the IRAP-Is (Albiston et al. 2008, Coutellier et al. 2011, Reed et al. 2011, Bild et al. 2013). This route of delivery causes the cannula to only pass through the motor and somtaosenory cortices, therefor it is possible that memory will not be affected, in contrast to previous studies. Pathological damage resulting from the surgery in terms of the level and spatial spread of immune cell activation and cytokine expression was also assessed.

To assess memory, the spontaneous alternation task (SAT) and novel object recognition task (NOR) were chosen. The SAT is largely a hippocampal dependent task (Lalonde 2002) and is a measure of spatial working memory, while the NOR is a measure of recognition memory and is dependent on a diverse number of brain regions (particularly the prefrontal cortices) including

the hippocampus (de Lima et al. 2006, Broadbent et al. 2010, Antunes et al. 2011). Both of these tasks were chosen as they are commonly used in the testing of nootropic agents and the NOR in particular chosen to provide a direct comparison with earlier studies investigating memory in response to surgical procedures.

2.2 Methods

2.2.1 Surgery

Male Sprague Dawley rats (270 – 310g, 7 – 8 weeks old) were housed in groups of 4 with *ad libtum* access to standard rat chow and water until time of surgery (All research conducted in this study was approved by the Monash University Animal Welfare Committee (MUAWC) under the Monash Animal Research Platform (MARP) 2011/117 application and performed according to the National Health and Medical Research Council of Australia "Code of practice for the care and use of animals for scientific purposes"). On the day of surgery, rats were anaesthetized with 5% (v/v) isoflurane before being placed in a Kopf stereotaxic frame and then maintained at 2% (v/v) isoflurane for the remainder of the surgery. Rats were implanted with an indwelling cannula (22 gauge from Plastics One; USA) in the right cerebral ventricle using the following flat skull coordinates; 0.8mm posterior to Bregma, 1.5mm lateral to the midline and 3.5mm ventral to the surface of the skull. The cannula was secured to the skull with a stainless steel screw and dental cement. During brain collection it was confirmed that the screw did not penetrate the dura. 1mg/kg intraperitoneal Meloxicam was given during surgery and rats supplied with 15mL of 200mg/kg paracetamol in 10% sugar water for two nights post-surgery as analgesia. Control rats (n = 22) received no treatment or anaesthesia.

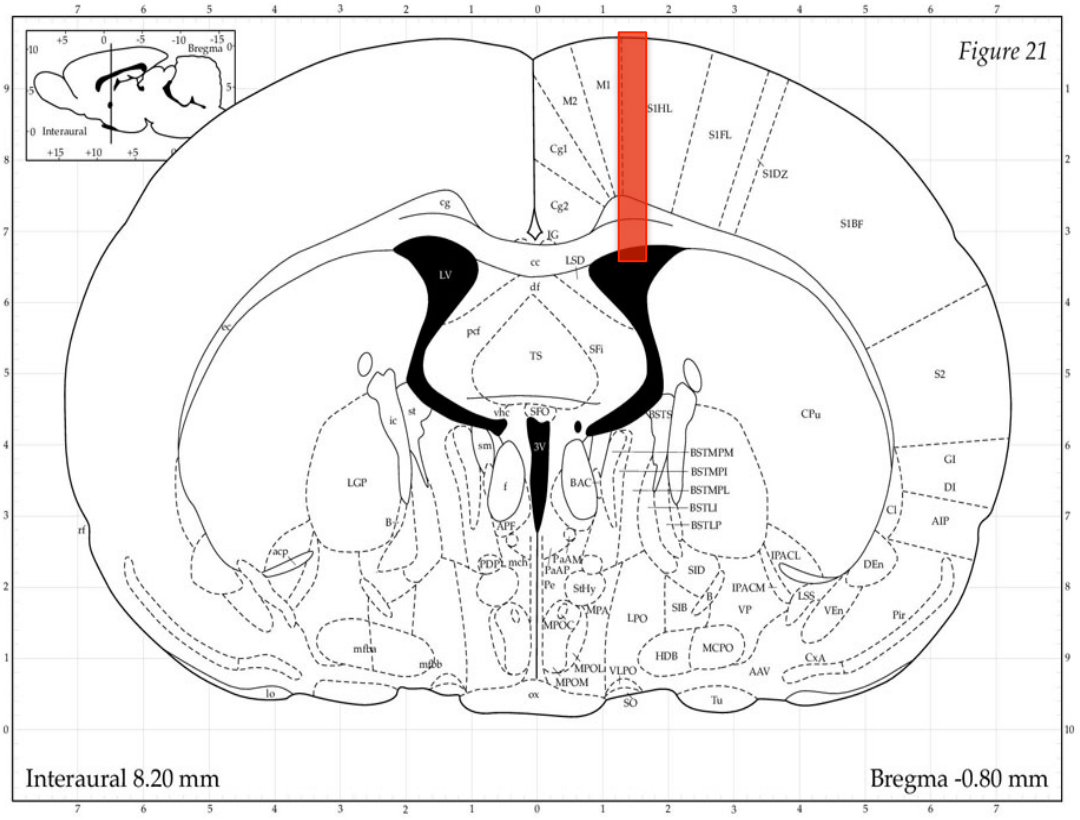


Figure 2-1 Placement of the indwelling cannula into the lateral ventricle (Image adapted from "That rat brain in stereotaxic coordinates" *Paxinos and Watson 1982*)

All rats (both control and cannulated) were individually housed post-surgery and handled daily for 5 minutes to familiarize them with human contact. All rats were tested in the spontaneous alternation task (to examine working spatial memory) on day 6-post surgery and the novel recognition task (to examine recognition memory) on days 7 and 8. Of these animals a smaller subset completed the elevated plus maze (to assess anxiety) on day 8. A second subset performed the rotarod task (to assess motor coordination) one day prior to surgery to establish a baseline and a second trial 6 days post-surgery to assess the effect of surgery. A third subset of animals instead performed the SAT two weeks post surgery to examine whether cognitive deficits may develop at longer intervals.

Prior to each behavioral task the rats were acclimatized to the testing room for 40 minutes. The testing apparatus was sprayed and wiped down with 80% ethanol before the rat began the task to remove olfactory cues. All tests took place in the same room with diffuse dim lighting; the testing area itself consisted of 3 white walls and a white curtain behind which the experimenter sat during the experiments. All behavior was recorded by a camera suspended above the testing arena and manually scored. Due to the presence of the cannula on the heads of the animals that had received surgery it was not possible to perform the experiments blinded, however, strict inclusion/exclusion criteria were in place in an attempt to reduce bias.

2.2.2 Confirmation of cannula placement

Correct cannula placement was confirmed by observing a dipsogenic response to angiotensin II (Johnson et al. 1975, Hoffman et al. 1976). Four days post-surgery rats received an injection into the lateral ventricle of 2µL of 25nM angiotensin II followed by 2µL sterile saline (to ensure complete delivery), placed back in their home cage and water intake measured every 5 minutes for 20 minutes. The cannula was deemed to be correctly placed if the rat drank a minimum of 5mL in the first 15 minutes. No rats were excluded under this criterion. To avoid possible memory enhancement by AngII, rats were tested at least two days after this test to ensure complete elimination of the compound.

2.2.3 Elevated Plus maze

The elevated plus maze consisted of four arms 70 (long) x 10 (wide) cm with two 'open' arms opposite each other having walls of clear Perspex 5 cm high and two 'closed' arms with 27 cm high grey Perspex walls. The floors were white laminate. The maze was elevated 85 cm above the ground. Rats were placed in the central platform facing one of the open arms, and behaviour was monitored for 5 minutes. The ratio of time spent exploring the open arms vs. the closed arms was used as a measure of anxiety.

2.2.4 Spontaneous Alternation Task

The plus maze had four arms measuring 75 (long) × 10 (wide) × 20 (high) cm and visual cues placed 1 m away from the center of the maze. The floor of the maze was white plastic while the walls were black. The test was performed by placing the rat in the center of the maze (all animals placed facing the same arm) and allowing 20 minutes of unimpeded exploration. The number and sequence of arm entries were recorded for calculation of a percent alternation score. An alternation consisted of the rat entering all four arms within the space of five arm entries (ADCAB would count as an alternation while ADCAD would not due to the rat not visiting arm B). Dividing the number of observed alternations in overlapping quintuplets by the number of possible alternations and multiplying the quotient by 100 calculated an alternation score.

2.2.5 Novel Object Recognition (NOR) Task

Prior to the acquisition trial, the rats were habituated for 30 minutes in the testing box (made from grey Perspex of dimensions 60 x 60 x 50 cm) in diffuse dim light and then returned to their home cage for 30 minutes. During the familiarisation trial, rats were placed in the box facing away from two identical objects (cylindrical glass jars) that had been secured to the floor in adjacent corners (with enough room to allow complete movement around them) of the box. Rats were allowed 5 minutes to explore the objects and exploration time recorded, with exploration defined as the nose being less than 2 cm from the object when facing the object and actively engaging with it (sniffing, paw touching etc.). Climbing on the object was not considered as exploration.

Here an inter-trial interval (ITI) of three hours was chosen to more closely match previous work investigating cognitive deficits following surgery (Frumberg et al. 2007, Hirshler et al. 2010). After the ITI of 3 hours, one of the familiar objects was replaced with a novel object made from the same material but of a different shape (glass jars, one cylindrical and one square). During this test trial the rats were given 2 minutes to explore the box. The recognition index was determined as the time spent exploring the novel object, minus the time spent exploring the familiar object, and then divided by the time spent exploring both objects. Rats were excluded from analysis if they spent less than 30 seconds exploring the objects during the familiarisation trial, or less than 5 seconds exploring the objects in the test trial. These times have previously been used within our lab and ensure that the rats explore the objects adequately. Under these criteria, 8 animals were excluded from the control group and 4 from the cannulated group.

2.2.6 Rotarod

The rotarod consists of a motorised cylindrical assembly around which there are 18 stainless steel rods that the rat is required to walk on. The mechanism is capable of rotating at a maximum speed of 30 revolutions per minute (rpm) (Ratek, Australia). For the animal to maintain an upright position on the rotarod, it had to grip the rods and keep "walking" as the rods rotated. Starting at 6 rpm the speed was increased by 1.5 rpm increments every 3 seconds until 30 rpm was reached. In the case that a rat fell off the device, or that it gripped the rods but did not walk so that it rotated with the device, rotation was stopped, the speed was decreased by 1.5 rpm, the rat was replaced on top of the rotarod and rotation was resumed. Rats were given three attempts to reach maximum speed and the fastest speed at which they could maintain an upright position on the rotarod for 3 seconds was recorded. Each rat was tested one day prior to surgery and again 6 days post surgery and the change between these scores used to assess the level of motor impairment.

2.2.7 Immunohistochemistry

Following performance of the NOR (eight days post surgery) rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg)

before being transcardially perfused via insertion of a needle into the left ventricle with ice-cold phosphate buffered saline (PBS; pH 7.3) and then ice-cold 4% (w/v) paraformaldehyde and 4% sucrose (w/v) in PBS. Animals were decapitated, the cannula removed and the brains rapidly harvested before being placed in the same fixative for 24-hours, cryoprotected by immersion in 30% (w/v) sucrose overnight at 4°C and then snap frozen in isopentane on dry ice and stored at -80°C.

Twelve-micron coronal sections were cut using a cryostat and thaw mounted onto gelatin-chrom-alum coated glass microscope slides. Sections were incubated with 10% (v/v) normal donkey serum (Sigma-Aldrich) in PBS with 0.3% (v/v) Triton-X-100 for 1 hour at room temperature to block non-specific binding of the antisera and permeablize the cell membranes. Sections were then incubated overnight at 4°C with the following primary antibodies; rat anti-GFAP to stain for astrocytes (Life Technologies), rabbit anti-IbA1 to stain for microglia (Serotech) and mouse anti-NeuN to stain for neurons (Life Technologies), all at a 1:500 dilution in PBS with 3% (v/v) normal donkey serum and 0.3% (v/v) Triton-X-100. Sections were then washed three times with PBS containing 0.1% (v/v) Tween-20 and the following fluorescent-conjugated secondary antibodies applied in PBS for 2 hours at room temperature; AlexaFluor 488 anti-rabbit and AlexaFluor 568 anti-rat (Introvogen). Sections were washed a final three times with PBS containing Tween-20 before being coverslipped with Dako fluorescence mounting medium containing 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) prolong gold (Life Technologies).

2.2.8 RT-PCR

Eight days post-surgery, rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (20mg/kg) until the heart just ceased beating. Eye and toe reflexes were confirmed to be absent before a cervical dislocation was performed, the cannula removed and the brain rapidly dissected on ice. For the cortical sample, tissue immediately surrounding the cannulation site was taken as well as tissue taken from the contralateral hemisphere in the corresponding location. The hippocampus was also dissected and divided into ipsi- and contra-lateral sides for the animals that had received surgery. For

control tissue, tissue was taken from the same points although the hemispheres were collected together in this case.

Total RNA was extracted from dissected tissues (25 mg) stored at -80°C using Trizol reagent (Life Technologies) according to the manufacturer's protocol. RNA quantity and quality were determined using A_{260}/A_{280} readings by NanoPhotometer (Implen). For RT-PCT, two microgram of RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For RT-PCR, the synthetized cDNA was used as a template for PCR reactions using TaqMan Fast Advanced Master Mix (Applied Biosystems) and appropriate TaqMan Gene Expression assay (Applied Biosystems). The TaqMan probes have the following identification number: 45S (Rn03928990_g1), GFAP (Rn00566603_m1), CD-11b (Rn00709342_m1), TNF-α (Rn00562055_m1), iNOS (Rn00561646_m1), IL-1B (Rn00580432_m1) and IL-6 (Rn01410330_m1). Relative quantification of gene expression was performed by comparative threshold (CT) method (calculated by the $2^{-\Delta\Delta CT}$ method). Changes in mRNA expression levels were calculated following normalization to the housekeeping gene 45S.

RT-PCR was performed by Vi Pham

2.2.9 Statistical analysis

Statistical tests performed in Grahpad Prism 6 and described under each figure as appropriate.

2.3 Results

2.3.1 Effect of cannulation on spatial and recognition memory

In order to evaluate the impact of a unilateral intracerebral ventricular cannulation on cognition, two separate behavioural tasks were chosen, the SAT to assess spatial working memory and the NOR task to assess object recognition memory. Each of these tasks was chosen as they are commonly utilized within the literature to assess potential nootropic agents, such as the IRAP-Is.

In the SAT, both groups performed above chance levels (> 40% alternation) when tested either one (Figure 2-2) or two (Figure 2-3) weeks post surgery. There was no difference in the alternations scores between control and cannulated rats, indicating that the cannulation did not impair spatial working memory. In the literature, the majority of animals are tested one-week post surgery; however there are cases of those examined at two weeks, hence the inclusion of the second cohort here. This work suggests that memory performance is stable across both weeks and does not develop with increased time. Of note is that the cohort tested at two weeks does have a slightly lower alternation score compared to the one-week cohort (Mean alternation score of 56 Vs. 67), however the variation within each group is small and this difference is not statistically significant. Lastly, testing was not performed earlier than one week as the animals were still recovering from surgery at this time, as this would introduce a confounding factor to the testing.

In the case of the NOR, both groups once again performed above chance (>0% recognition index) and there was no difference between the control and cannulated animals (Figure 2-4), indicating that there was no impairment to recognition memory caused by the cannulation. The absence of deficit in the SAT suggests no impairment to hippocampal function and the absence in the NOR task supports and suggests the same of frontal cortices.

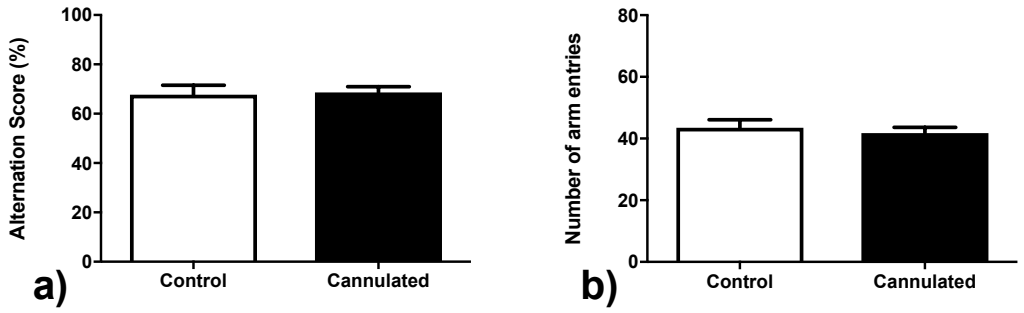


Figure 2-2 Comparison of performance in the spontaneous alternation task by cannulated and surgically naïve rats one week following surgery

(a) Both groups performed above chance and there was no difference in the performance of rats that had either received an i.c.v cannulation (n = 24) or were surgically naïve (n = 22) (p = 0.83) (b) There was no difference in the number of arm entries between either group (p = 0.59).

Data represented as mean ± SEM, P value determined by two-tailed unpaired t-test

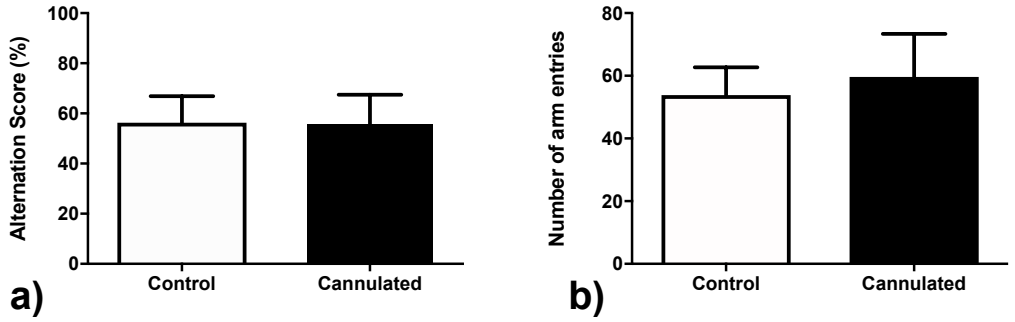


Figure 2-3 Comparison of performance in the spontaneous alternation task by cannulated and surgically naïve rats two weeks following surgery

(a) Both groups performed above chance and there was no difference in the performance of rats that had either received an i.c.v cannulation or were surgically naïve (n = 8 / group) (p = 0.91) (b) There was no difference in the number of arm entries between either group (p = 0.38).

Data represented as mean ± SEM, P value determined by two-tailed unpaired t-test

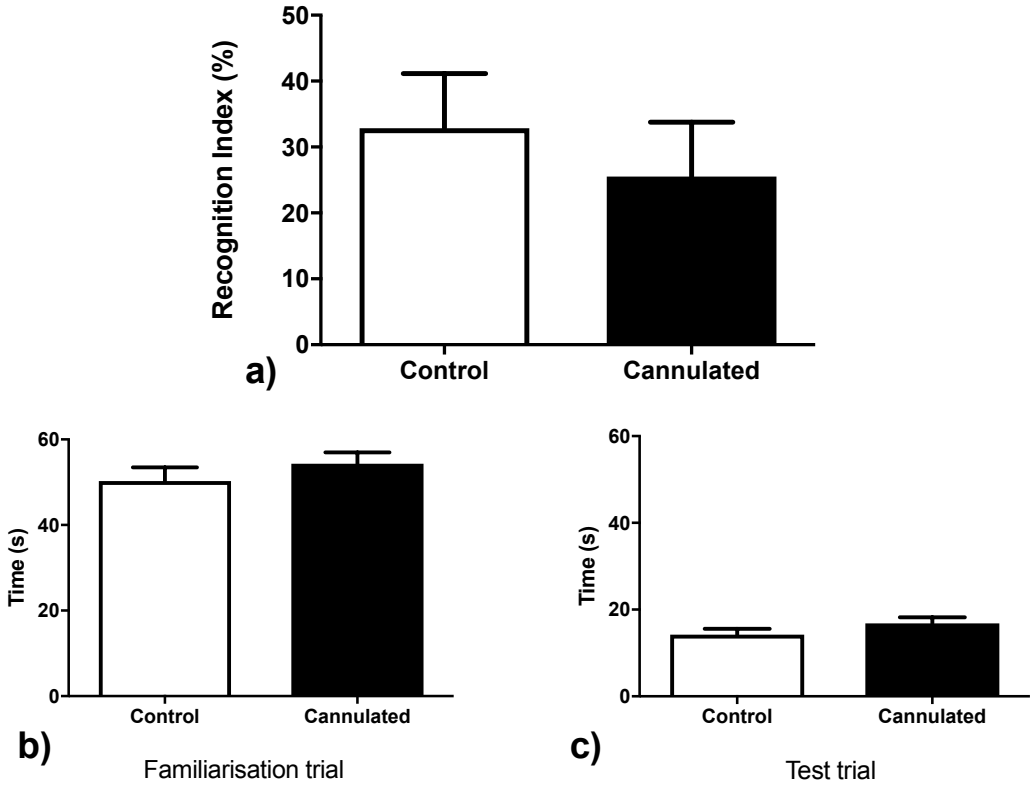


Figure 2-4 Comparison of performance in the novel object recognition task by cannulated and surgically naïve rats

Both the cannulated (n = 20) and naïve (n = 14) animals displayed a preference for exploration of the novel object, indicating they were able to recall the familiar object and there was no difference in the recognition index between each group (p = 0.54). There was no difference between groups in the amount of time spent exploring the objects in either the (b) 5-minute familiarization trial (p = 0.5) or (c) 2-minute test trial (p = 0.22)

Data represented as mean ± SEM, P value determined by two-tailed unpaired t-test

2.3.2 *Effect of surgery on animal health*

As the cannulation surgery represents a stressor to the rats, body weight was recorded in the week following surgery. Control rats demonstrate a steady increase in body weight from an average of 325g to 342g. The cannulated rats showed an initial decrease of 11g in the two days following surgery, before body weight returned to baseline by the time the behavioral testing (Figure 2-5), indicating a return to health. Body weight of the cannulated animals was also not significantly different from the control animals at any point. The surgery may have also caused the rats to be become anxious, which in turn would impact performance. To combat this animals were handled daily and as seen in the elevated plus maze, both groups spent an equal amount of time in the open and closed arms indicating no differences in anxiety (Figure 2-6) (Greater time spent in the closed arm indicates the animal is too anxious to explore the open arms).

Considering that the cannula descended through the motor cortex before reaching the ventricle there was also concern that the motor function of the animals could be affected. Using the accelerating rotarod, a slight impairment of fine motor coordination was noted, with a decrease in max running speed score of 1.2 (-12% from initial score) (Figure 2-7). Impairment was deemed as slight here as the animals' gross movement appeared unaffected when observed both in the home cage and while performing the memory tasks. Confirming this, during performance of the memory tasks, the number of arm entries in the SAT (Figure 2-2b) and the total exploration time in the NOR (Figure 2-4 b,c) were the same between both groups.

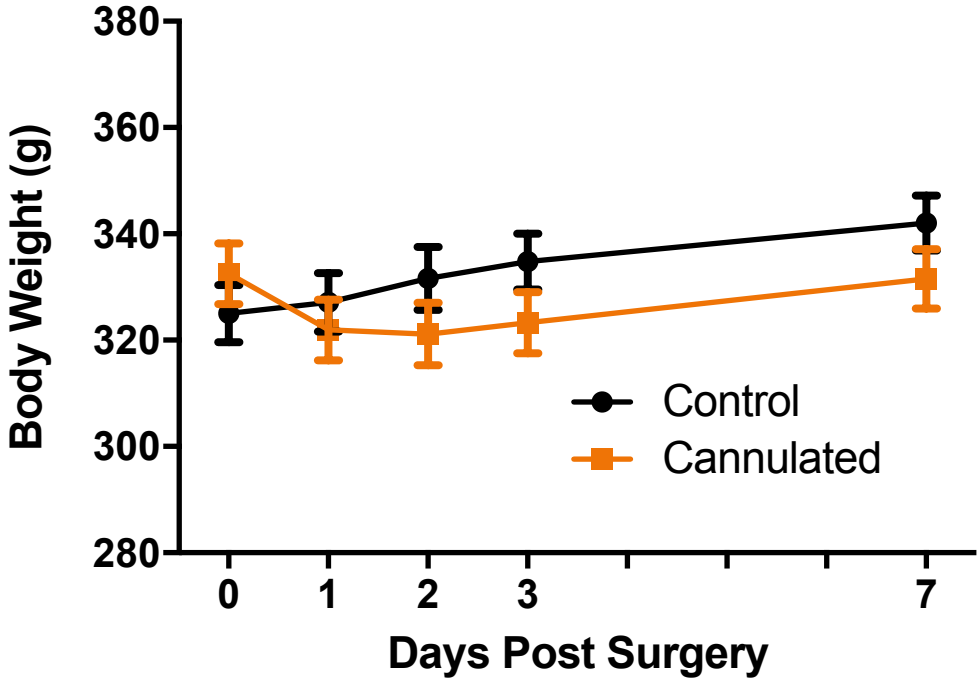


Figure 2-5 Body weight change following surgery in rats receiving the i.c.v surgery

Rats that underwent surgery showed an initial decrease in body weight in the two days following surgery, however they gain weight after this point and return to pre-surgery levels. Control rats continue to gain weight throughout the testing period. There was no significant difference in the body weights due to surgery ($p = 0.44$, $F(1,46) = 0.5855$)

Data represented as mean ± SEM, P value determined by 2-way ANOVA with Sidak correction and time and surgery as covariates

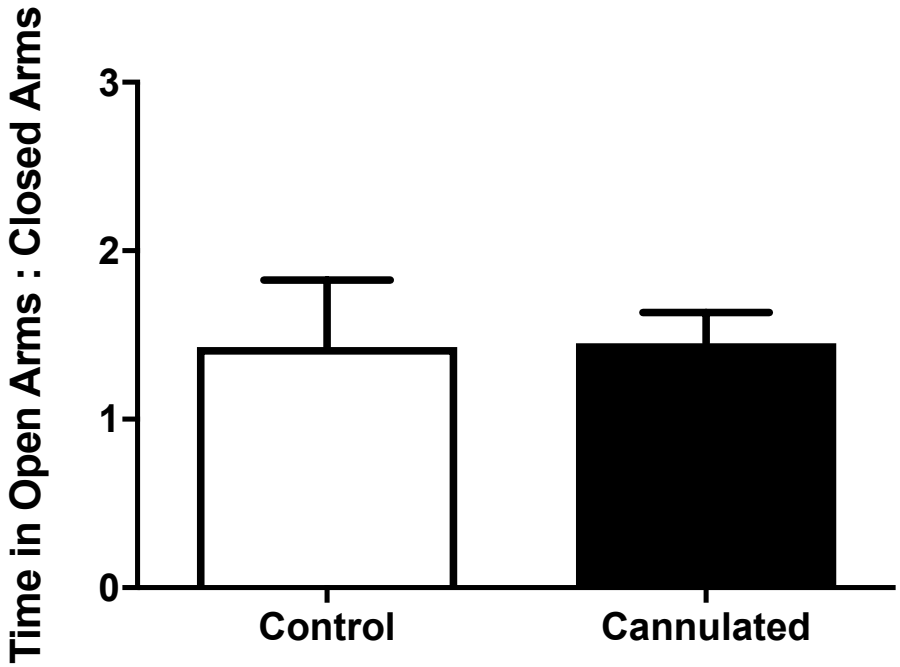


Figure 2-6 Ratio of time spent in the open vs. closed arms of the elevated plus maze

Both the surgically naïve (n = 8) and cannulated rats (n = 12) spent an equal amount of times in the open and closed arms (p = 0.95).

Data represented as mean ± SEM, P value determined by two-tailed unpaired t-test

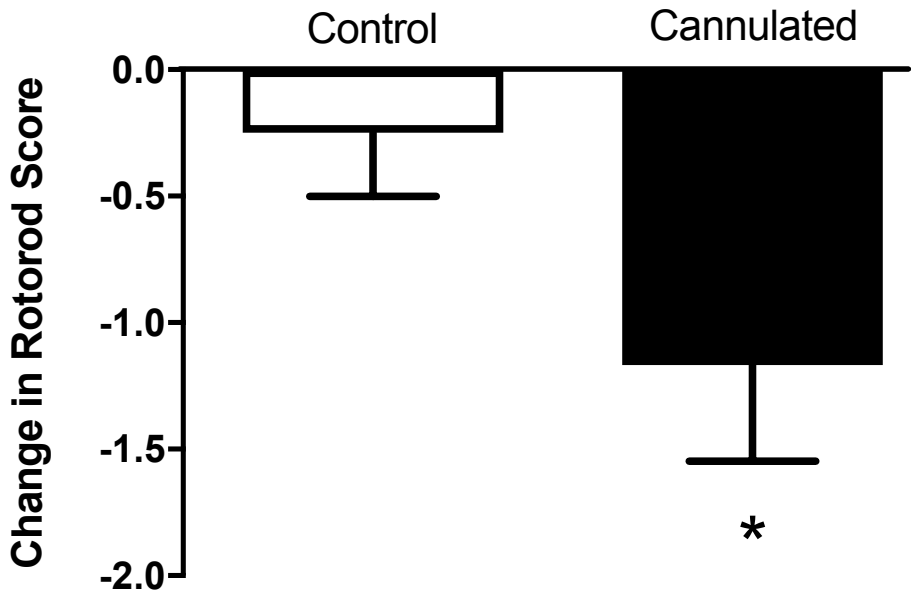


Figure 2-7 Change in max running speed on the rotarod task by control and cannulated rats.

All rats were able to reach the max speed of 30 rpm prior to surgery. When tested post surgery cannulated rats (n = 6) showed a greater reduction in motor coordination in the second trial compared to control animals (n = 6) (p = 0.038).

Data represented as mean ± SEM, as it was predicted that the control animals would not show a change in their score the P value determined by one-tailed unpaired t-test with Welch's correction

2.3.3 Immunohistochemical analysis of brain sections 8 days following surgery

To examine the activation of the brains immune cells, staining was performed against proteins heavily localized to the specific cells, GFAP for astrocytes (Eng et al. 2000) and IbA1 for microglia (Ito et al. 1998). Examining the damage resulting from the cannula implantation at a cellular level, it was noted that there was a population of activated astrocytes and microglia at the base of the cannula path, as indicated by the increased GFAP and IbA1 staining respectively, while there was also a possible thin layer of scar tissue formed along the tract of the cannula insertion (indicated by the *) (Figure 2-8 a,c). Importantly this immune cell activation did not spread laterally from the cannulation site and there was no evidence of inflammation on the contralateral side of the cortex (Figure 2-8 b,d). The characteristic activated/reactive state of both astrocytes and microglia can be observed in the higher magnification images (Figure 2-9 a,b). Notably, there is little overlap in the staining of GFAP and IRAP (Figure 2-9a), contrasting with the earlier reported work from our lab (*Yeatman,* unpublished). While previous evidence has noted IRAP expression in activated astrocytes following injury, particularly cortical stab wounds (*Fernando,* unpublished), here IRAP expression appeared restricted to neurons.

Staining for NeuN was also performed in order to visualise the extent of neuronal loss surrounding the cannula. While there is a clear area of cell death at the base of cannula (corresponding to the population of activated immune cells), NeuN staining can be seen right up to the edge of the cannula tract (Figure 2-10). The pattern of immune cell activation and cell death suggests that any deficit is likely restricted to the cortical area immediately at the base of the cannula path (part of the motor cortex) and does not extend in the hippocampus, in line with the slight impairment seen to motor coordination but lack of deficit observed in the two memory tasks.

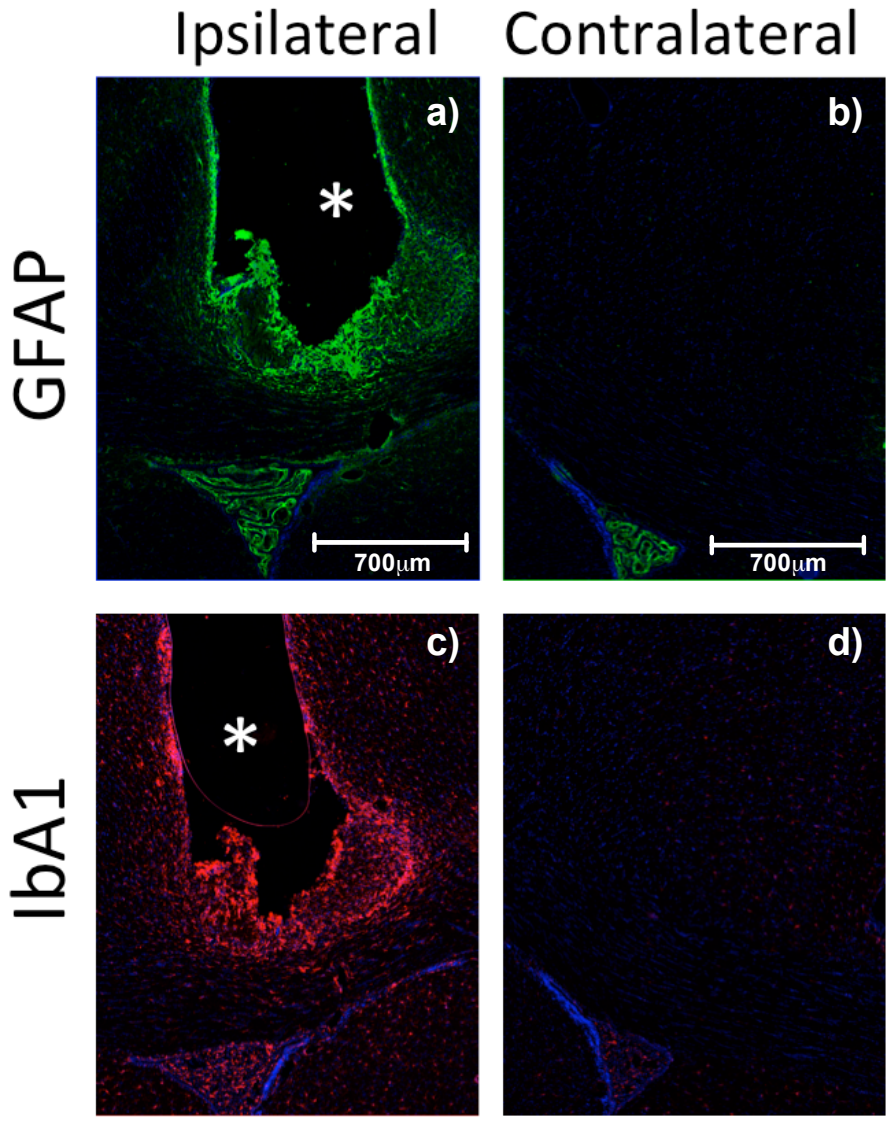


Figure 2-8 Immunohistochemistry for immune cells around the cannulation site

*Population of astrocytes (GFAP in green) and microglia (Iba1 in red) in the brains of rats that had received an ICV cannulation. (Left) the area immediately surround the cannulation, * indicates the path of the cannula (Right) the contralateral hemisphere*

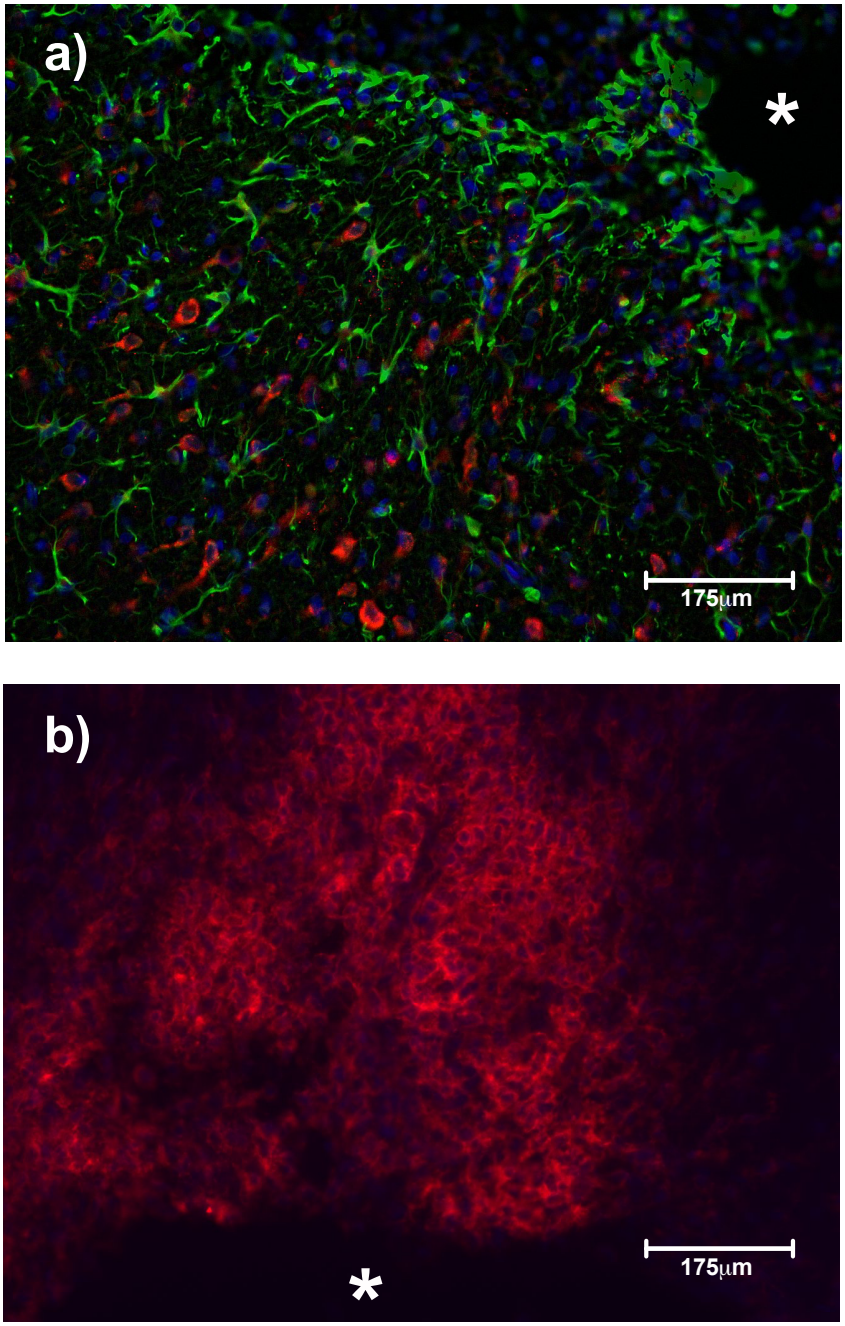


Figure 2-9 Immunohistochemistry, 20x Magnified view

*Increased magnification of the base of the cannula path demonstrating the expression of IRAP and the activated phenotype of the immune cells. (a) GFAP in green and IRAP in red. There is little to no overlap between the expression of GFAP (which shows typical astrocyte staining) and IRAP (which shows classical neuronal expression) (b) IbA1 staining indicating microglia in red. * indicates the path of the cannula in each image*

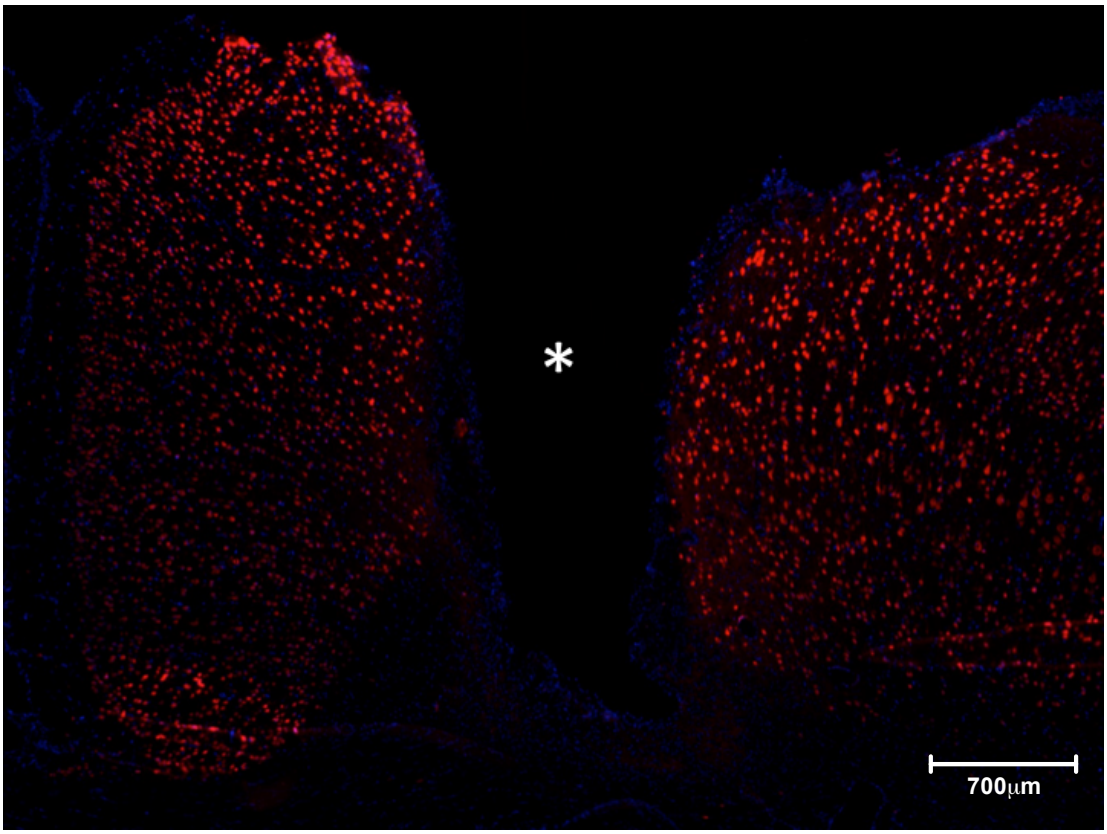


Figure 2-10 Immunohistochemistry for live neurons (NeuN in red) in the brains of rats that had received an ICV cannulation

*Neuronal staining can be seen right up to the very edges of the cannula path, with only the cannula base appearing to have neuronal death. * indicates the path of the cannula through the cortex.*

2.3.4 Real-time PCR following cannulation surgery

Using RT-PCR on tissue samples collected eight days post surgery, an upregulation of both GFAP (marker of astrocytes) and CD-11b (marker of microglia) mRNA in the cortex of cannulated animals in the area directly surrounding the cannula tract was noted, while there was no change in the contralateral hemisphere or hippocampus (Figure 2-11 e,f), in agreement with the immunohistochemistry observed in (Figure 2-8).

Examining the levels of inflammatory mediators, no upregulation of the major cytokines TNF-α or IL-6 was noted in either cortical hemisphere or hippocampus (Figure 2-11 a,b). The lack of upregulation in hippocampus is particularly important, highlighting again that any damage resulting from the surgery appears to have spared this crucial memory region. While IL-1β and iNOS both show large elevations in the cannulated cortex (Figure 2-11 c,d) these results are not significantly different and are due to an animal showing exceedingly high expression as evidenced by the box-and-whisker plot (Figure 2-12 c,d). This rat did not show outward signs of infection nor did its body weight reflect ill health thus it is unlikely that it was suffering from a systemic infection. It is possible that a localised infection took place, although there were no visible signs at the time of brain collection. It is unclear why only IL-1β and iNOS are upregulated, but not TNF-α and IL-6. This could reflect that TNF-α and IL-6 are more relevant earlier in the cascade; while IL-1β and iNOS are still active at the time that rat was killed.

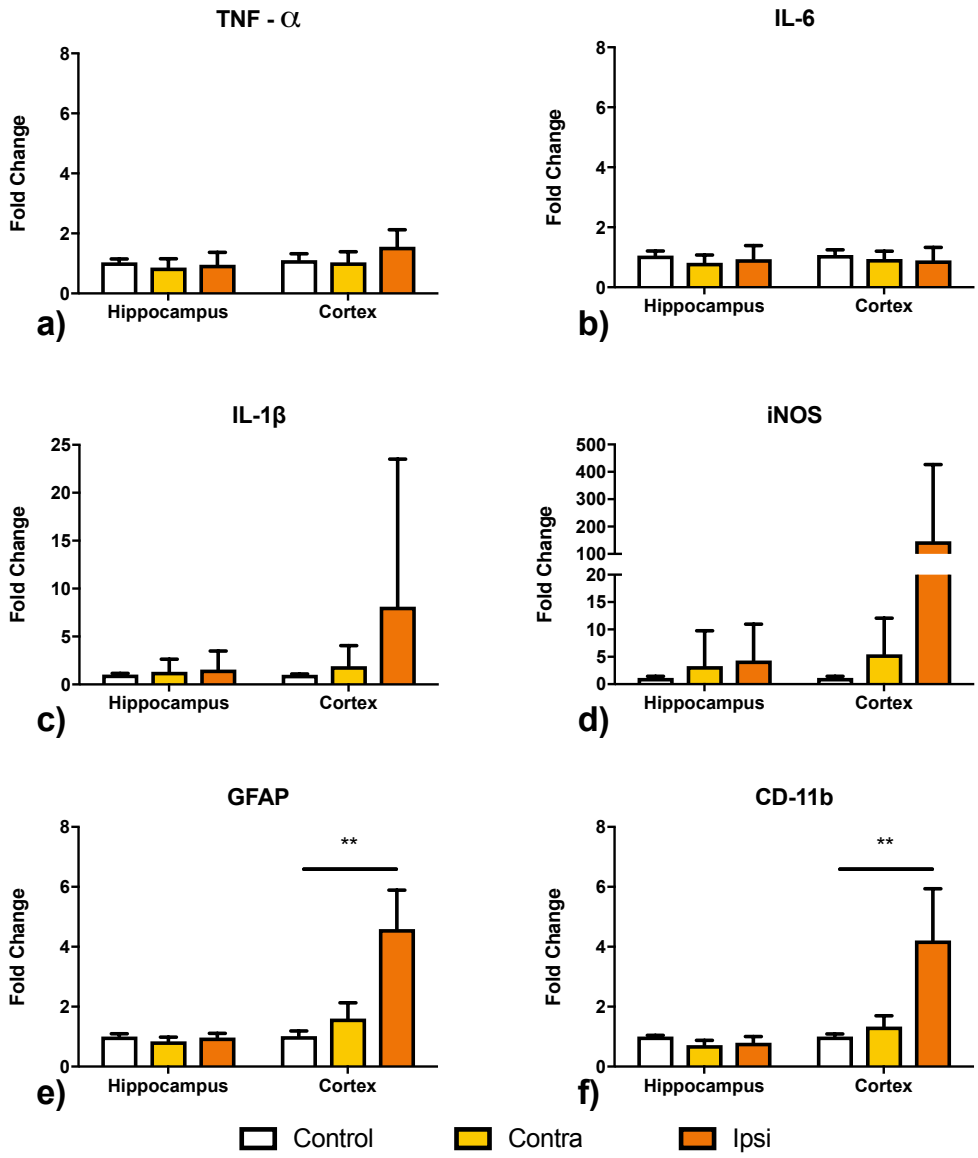


Figure 2-11 mRNA expression of cytokines and immune cells in cannulated rats

(a – d)There was no significant upregulation in either the hippocampus or the cortex for any of the inflammatory cytokines, TNF-α, IL-6, IL-1β or iNOS. GFAP and Cd11b mRNA expression was elevated in the area of cortex immediately surrounding the cannulation site. Gene expression was normalized against the house keeping gene, 45S. n = 5 controls and 9 cannulated animals.

*Data presented as mean ± SEM, ** P < 0.01, 1-way ANOVA with post-hoc Bonferroni (separate for each cortex and hippocampus)*

Work performed by Vi Pham

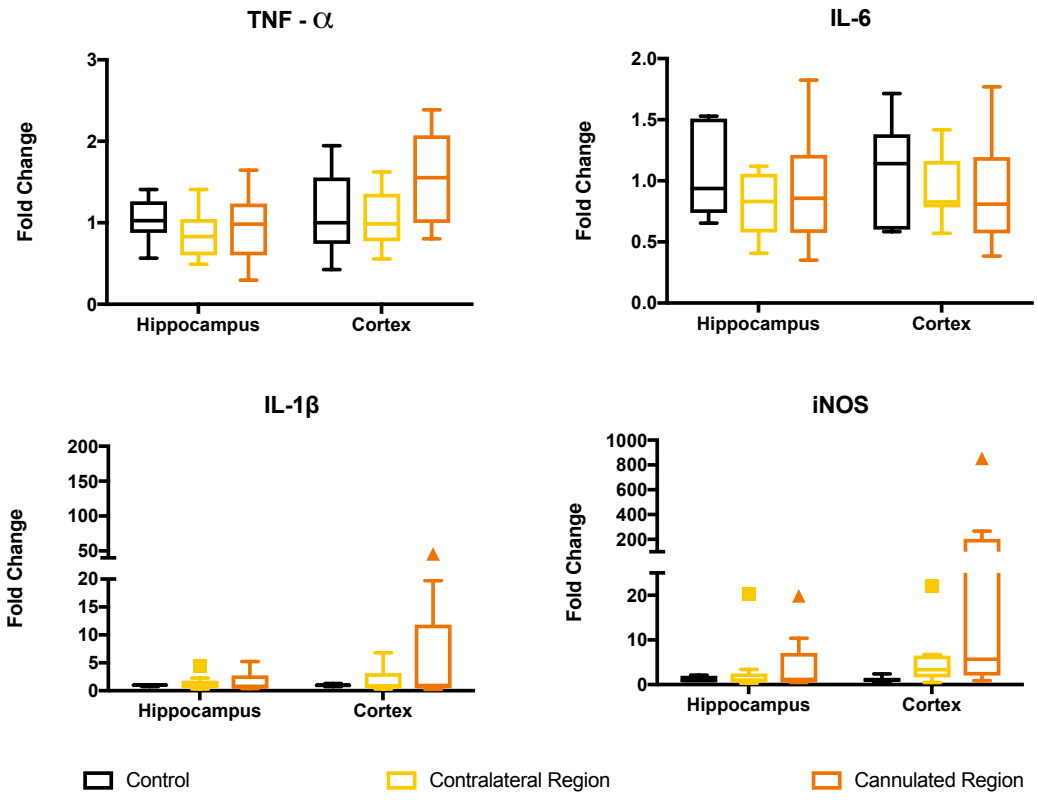


Figure 2-12 Tukey box-and-whisker plots of mRNA expression of cytokines cells in cannulated rats

While the expression of TNF- α *and IL-6 are consistent across all animals, there are divergent animals in regards to iNOS and IL-1* β *expression. In each case there is an outlier with expression well outside the range of the others. The animal with the elevated expression is the same in both the IL-1* β *and iNOS groups. Gene expression was normalized against the house keeping gene, 45S*

2.4 Discussion

This work sought to characterize the behavioral and cellular consequences of the implantation of a chronic indwelling cannula into the lateral ventricle of the brain. This serves to inform as to whether the IRAP inhibitors are enhancing normal memory or instead rescuing a deficit in cognition imposed by the surgery itself. The precise physiological role of IRAP remains unknown and by understanding the paradigms in which it is examined, indications as to the mechanism governing how the IRAP-Is enhance memory may be found. This is also relevant for the field at large with many nootropic agents delivered into the brain at this time point via this method in pre-clinical studies (Parsons et al. 1992, Lee et al. 2004, Albiston et al. 2008). In this work, while a slight impairment of motor coordination was noted, there was no negative effect of surgery seen on two common memory tasks and there appeared to be only a restricted inflammatory response around the cannulation site.

2.4.1 Comparison with previous behavioral studies

A number of studies have examined the impact of a range of surgical procedures on brain function, however only two have explicitly examined whether memory is adversely affected (Frumberg et al. 2007, Hirshler et al. 2010). Both of these studies detected impairment in recognition memory in contrast to the work presented here. In the first, a striatal microdialysis cannulation was performed (Frumberg et al. 2007) while the second was a bi-lateral implantation of electrodes into the subthalamic nucleus, which also caused neuroinflammatory responses in several regions of the brain including the entorhinal, parietal and frontal cortices (Hirshler et al. 2010).

One of the key differences between these and the current study is the relative severity of the surgical procedure. The study by Frumberg and colleagues descended 6 mm below the surface of the skull and deep into the caudate, while Hirshler and colleagues performed bi-lateral electrode insertions beginning 3 mm anterior to bregma at a 38° angle and ending 3 mm posterior, passing through prefrontal cortex, striatal areas and the thalamus before terminating in the subthalamic nucleus. In both cases, areas implicated in memory and learning may have been damaged by the cannula/electrode

passage and/or subsequent inflammation. By comparison, the i.c.v cannulation used here only descended 3.5 mm into the brain and passed through motor/somatosensory cortex. Thus while direct comparison is difficult, it could be argued that fewer areas (specifically those associated with memory) are damaged by this procedure, which could account for the lack of cognitive deficit seen in this work.

Both previous studies performed pre- and post-surgery behavioural tests (Frumberg et al. 2007, Hirshler et al. 2010), while the present study only involved testing post-surgery. Pre-testing was deemed unnecessary given that the control animals did not receive either anesthesia or surgery of any kind and thus reflect the animals in an unchallenged state. Secondly, testing of animals multiple times in the same behavioural task introduces an element of learning that is avoided in this study, particularly important in the case of the SAT, which is reliant on working memory.

Age of the animals is unlikely to have been a confounding factor when comparing between studies. Similar to the work by Frumberg and colleagues, rats in the current study were two months of age; while in the study by Hirshler and colleagues they were four months of age. Both these ages represent early adulthood of the animals and it is unlikely that recovery from cortical insult would differ between them.

Also of note is that neither the previous studies, nor the current work, featured a volume infusion in addition to cannulation. Given that the ventricles are a fluid filled space, as opposed to a neuron dense region such as the striatum, it is unlikely that the addition of a small volume (2μL being common for the delivery of IRAP-Is (Albiston et al. 2008)), would cause structural damage and adversely affect cognitive performance. However, the possibility cannot be ruled out, particularly if a constant infusion is performed, and as such future studies should be directed towards examining this.

2.4.2 ICV cannulation causes minimal inflammatory response

As neuro-inflammation has previously been linked to cognitive deficits (Butler et al. 2004, Sparkman et al. 2006, Ren et al. 2011), immunohistochemistry and RT-PCR was performed to quantify the spread of

immune cell activation and the levels inflammatory cytokine expression. In this work, activation of astrocytes and microglia was restricted to the area immediately surrounding the cannulation site and there was no upregulation of either TNF-α or IL-6 (two major cytokines). This parallels evidence from both stroke (Berti et al. 2002) and trauma (Taupin et al. 1993) studies demonstrating that expression of both TNF-α and IL-6 is decreasing or has returned to baseline levels seven days post-insult. While there appears to be very large response from both IL-1β and iNOS this is not significant and is due to an animal showing exceedingly high expression levels. Both these and the histological results imply a very localised inflammatory response that does not extend beyond the immediate site of cannulation in the motor cortex. The limited spread of the inflammatory response is also supported by the relative lack of neuronal cell death, apparent only at the base of the cannulation and is also in line with previous studies that have examined the cellular response to implanted devices (Kelsey et al. 2012). While previous reports from our lab have demonstrated that IRAP may be expressed in activated astrocytes (*Fernando, Yeatman,* unpublished*)* this was not observed in the current work.

2.4.3 The effect of isoflurane

While one of the more common anesthetics used in animal surgeries, isoflurane has been linked to memory deficits in rodents (Culley et al. 2003, Culley et al. 2004), though this has been disputed by others (Crosby et al. 2005, Zhu et al. 2010). The current study chose to examine the surgical procedure as a whole, rather than just the effect of the cannulation, thus the choice to not subject control animals to anesthesia could be a point of contention. This would only have become an issue in the case that a memory deficit had been observed, in which case it would not have been clear whether it was caused by damage resulting from the cannula insertion, or by the isoflurane anesthesia. While not specifically designed to investigate the effect of anesthesia, this work does support the notion that there is no lasting memory impairment caused by isoflurane. However this could also be due to the relatively short duration of exposure, with surgery lasting only 25 – 30 minutes, in contrast to most previous studies that examined exposure times of at least 2 hours.

2.4.4 ICV cannulation implications for cognitive enhancement by IRAP inhibitors

The evidence presented here is exciting when considering the memory enhancement seen in response to IRAP inhibition, such as by AngIV (Braszko et al. 1988, Wright et al. 1999) and HFI-419 (Albiston et al. 2008). Previous evidence has shown that IRAP appears to be upregulated during various pathological states (Shibata et al. 2005), plays a role in antigen cross-presentation (Saveanu et al. 2009) and could play a role in neuroinflammation given its expression in activated astrocytes *(Fernando, Yeatman,* Unpublished*)*. In this work IRAP staining was not seen in astrocytes, even in the area immediately surrounding the insult. Further, as the i.c.v surgery does not impair baseline memory it suggests that IRAP-Is are enhancing normal memory rather rescuing a deficit. If the IRAP inhibitors were only able to rescue a deficit state, once work progressed to peripheral administration it would be unlikely that cognition would be enhanced as there would be not a surgery-induced deficit to correct. From a clinical aspect this is also promising, the ability to enhance normal memory suggests that IRAP inhibition could be a novel symptomatic treatment for any disorder with a cognitive deficit (or even age related cognitive decline) and is not restricted to a single disease process.

An argument could be made that the work presented here only investigated very young rats as opposed to older and transgenic AD mice that will be assessed later in the work. Following from this, it could be suggested that while the IRAP-Is may enhance memory in these 'unchallenged' animals, they would have no effect in the older animals and those that have accumulated transgene damage (as in the case of the AD transgenic mice for example). However, the purpose of this study was to determine the likely mechanism of IRAP-I action, true cognitive enhancement vs. restoration of a deficit and use this to drive the direction of further chapters. Nonetheless, the possible efficacy of the IRAP-Is in the AD mouse models is assessed in chapter 4.

This data also means that predictions can begin to be made about the likely mechanism of cognitive enhancement produced by IRAP inhibition. Given its enhancement of normal memory there are two likely avenues; elevated glucose uptake or altered expression of MMPs. IRAP is closely localised with the

inducible glucose transport $GLUT_4$ (Fernando et al. 2008) and inhibition has already been demonstrated to be able to enhance glucose uptake (Albiston et al. 2008). In turn, glucose loading is well documented for its ability to enhance cognition (Kopf et al. 1994, Smith et al. 2011, Glenn et al. 2014). Data from our collaborators also implicates IRAP inhibition as being able to alter the MMP/TIMP balance, key regulators of spine growth and cognition (*Unpublished data, Heuy Wen Lee*). Both of these avenues will be explored in the following chapter.

2.5 Conclusion

The current study demonstrates that the implantation of an indwelling cannula into the lateral ventricle does not adversely affect cognition. While an inflammatory response may be mounted following surgery, it appears to be spatially restricted around the site of the cannulation as evidenced by the limited spread of immune cells and lack of cytokine upregulation. Importantly, this means that cognitive enhancing effects of IRAP inhibition to date has been due to an enhancement of normal memory, rather than rescue of a deficit and begins to inform as to the possible mechanisms by which IRAP inhibition is acting. Perhaps more importantly it means that these compounds have the ability to enhance cognition across a variety of disease states where a cognitive deficit is a factor as well as acting as true nootropic agents.

Chapter 3

IRAP inhibitors mediate increases in dendritic spine density by enhancing glucose uptake through GLUT4

3.1 Introduction

As demonstrated in the previous chapter, the i.c.v cannulation surgery used to deliver IRAP-Is into the lateral ventricles of the brain does not induce a cognitive impairment in the animals. This suggests that the cognitive benefit of IRAP-I treatment is not due to rescue of a deficit, but rather an enhancement of 'normal' memory. This means that the current lead synthetic candidate, HFI-419, having already been validated as a cognitive enhancer after improving memory in the spontaneous alternation and novel object recognition tasks (Albiston et al. 2008) can be termed a true nootropic. Moving forward, identification of the mechanism governing this enhancement of memory is key to establish in order for the development of the IRAP-Is to move beyond preclinical stages. Additionally, whether other novel synthetic IRAP-Is can also produce a similar cognitive enhancing effect is also of interest in order to confirm a class effect.

A current hindrance to the development of the IRAP-Is is the lack of a high throughput screen. This is partly due to the bell shaped (or, inverted U-shape) dose response that is observed in response to IRAP-I treatment (Benoist et al. 2011, Diwakarla et al. 2016) and is a feature shared by many potential nootropic agents (Ramanujam 2014). This means that doses that are either too low or too high fail to facilitate cognitive enhancement and in the cases of the IRAP-Is, often only a single dose proves to be efficacious, 10-fold higher or lower doses produce no response. This presents a difficult problem when using behavioural testing as a screen for novel IRAP-Is that can potentially enhance cognition due to the time taken to first perform surgery on the animals, allow for recovery time and then conduct the tasks themselves. Combined, this means that high through-put of novel compounds as well as determination of a mechanism is particularly difficult to achieve.

Further compounding this, as demonstrated in the case of the aryl sulfonamides from our collaborators, not all IRAP-Is may produce the same biological effect. In this case, three compounds demonstrated similar IC_{50} values for IRAP inhibition, yet only two of the compounds promoted changes in dendritic spines (Diwakarla et al. 2016) and while this is relatively easy to discover in an *in vitro* assay, it would take extensively longer to determine in

behavioral memory assays. As the IRAP-Is are still in early preclinical testing this severely hampers screening and characterization of new compounds and highlights the need for a sensitive *in vitro* bioassay to determine which inhibitors are likely to effectively enhance memory.

One of the best cellular correlates of memory are dendritic spines, small protrusions from the dendrites of neurons that serve as contacts with neighboring axons and contain all of the molecular machinery required for synaptic plasticity and long-term potentiation, i.e. the storage of memories. There is a close correlation between dendritic spine density and learning *in vivo* that has been demonstrated in a number of behavioral studies such as passive avoidance (O'Malley et al. 1998), water maze tasks (Moser et al. 1994, O'Malley et al. 2000), fear conditioning (Lai et al. 2012) and repetitive motor tasks (Fu et al. 2012). Dendritic spines are easily observable in culture and the macrocyclic and arylsulfonamide derived IRAP-Is have already been demonstrated to enhance dendritic spine density in primary hippocampal cell cultures using a protocol that I developed in collaboration with our partners in Sweden (Diwakarla et al. 2016a, Diwakarla et al. 2016b), although as yet these inhibitors have not been shown to enhance memory *in vivo,* thus whether a positive result in the spine assay correlates to improved cognition remains unknown. The initial aims of the current work were to therefore;

1) Validate the use of the spine assay by demonstrating increased spine growth with the same inhibitors that are able to enhance cognition.

2) Investigate novel IRAP-Is for potential cognitive enhancing effects

As will be discussed, pilot work that focused on testing of novel IRAP-Is in memory paradigms highlighted the aforementioned issues associated with behavioural testing. Further, although the dendritic spine assay is faster than using behavioural tests as a screen for novel IRAP-Is, there is still significant delay owing to need for the investigator to manually count spines across a range of doses. Ideally, discovery of the mechanism underlying spine growth itself would allow for development of a high-throughput assay. There are two likely mechanisms by which inhibition of IRAP leads to an enhancement of memory;

- By increasing neuronal glucose uptake

  And/or

- By altering the activity of the matrix-metallo-metalloproteinases (MMPs)

Modulation of both of these systems has been demonstrated to improve memory and increase dendritic spine density. Oral glucose loading can enhance cognition in humans (Smith et al. 2011) and from a mechanistic standpoint, glucose itself can activate mammalian target of rapamycin (mTOR), a key step in promoting the growth of mushroom spines (Dash et al. 2006). IRAP and the inducible glucose transporter GLUT4 are closely associated in neurons (Fernando et al. 2008) and while HFI-419 has already been demonstrated to enhance glucose uptake in hippocampal slices (Albiston et al. 2008), this has not been demonstrated in primary neurons in culture.

The MMPs are a family of enzymes that are widely distributed throughout the body and are responsible for the degradation of extracellular matrix proteins. Within the brain, this breakdown of the extracellular matrix releases dendritic spines from confinement and allows changes to their morphology, crucial in the development of mushroom spines (Oray et al. 2004). MMP9 is regarded as the most important in the brain, with inhibition of its activity impairing memory (Hiroyuki Mizoguchi 2010) and overexpression having a positive effect (Fragkouli et al. 2012). Treatment of trophoblasts with angiotensin IV leads to a decrease in the activity of MMP-9 (Cohen et al. 2008), however an interaction has not been noted in the brain.

This lead to the main focus of the current study, which sought to;

3) Investigate the mechanism of action governing the enhancement of dendritic spine growth,

4) Use this information to establish a sensitive and rapid *in vitro* bioassay for IRAP-Is that are likely to enhance cognition *in vivo.*

To achieve this, primary cultures of hippocampal neurons were grown and treated with various IRAP-Is as well as inhibitors of both glucose uptake and MMP-9 and the effect on spine density was examined. The results of this in turn drove the development of a bioassay for novel cognitive enhancing IRAP-Is.

3.2 Methods

3.2.1 Surgery and behavioural testing

Implantation of the cannula into the lateral ventricle, confirmation of placement and performance of the spontaneous alternation task were performed as described in the previous chapter. In this case however, animals were also administered either vehicle or drug to investigate the cognitive enhancing effects of the IRAP-Is. Using a protocol well established within our lab (Albiston et al. 2008), 5 minutes prior to the behavioural task the opening of the cannula was swapped with 80% ethanol before the rats were injected with 2µL of either vehicle (1% DMSO) or the appropriate dose of IRAP inhibitor, followed by 2µL of sterile saline to ensure complete delivery of the drug and returned to their home cage until the beginning of the task. All doses of IRAP inhibitor initially dissolved in 100% DMSO before dilution in sterile saline to specified concentration.

3.2.2 IRAP Inhibitor classification

This study made use of three IRAP-Is; the lead compound HFI-419 (IC_{50} = 0.49 µM) and two novel compounds SJM-164 (IC_{50} = 2 - 4 µM) and VR-006 (IC_{50} = 1.14 µM). SJM-164 was chosen as a structurally distinct benzopyran inhibitor of IRAP to test for a class effect, while VR-006 was chosen as it is the parent compound of a recently developed series of aryl sulfonamide based inhibitors used by our colleagues in Sweden.

In terms of bioavailability, the currently available data is not consistent across the compounds. Following oral administration (3mg/kg), despite 100% intestinal permeability (CACO-2 assay) HFI-419 is rapidly broken down in the plasma with 14.6 ng/mL found 20 minutes post administration and none detectable in the brain. It also shows poor metabolic stability on both microsomes (~30%) and hepatocytes (~5%). SJM-164 shows only 65% intestinal permeability but its bioavailability could not be determined as the compound could not be separated from either plasma of brain samples. It did

however show more favorable metabolic stability, ~82% on microsomes. VR-006 has been demonstrated to have good stability after two hours in plasma.

Profiling performed by CEREP against 110 targets shows that the benzopyran inhibitors demonstrate high specificity for IRAP. HFI-419 displays very low binding of human adenosine 2A receptors while SJM-164 was not seen to bind to any of the targets. Unfortunately specificity profiling for VR-006 has not been performed.

3.2.3 Culture of Primary Hippocampal Cells

Embryonic day 17 - 18 Sprague-Dawley pups were obtained from pregnant dams killed via isoflurane overdose. The hippocampus was dissected out in Earle's Balanced Salt Solution (Gibco) on ice under a dissecting microscope. Dissected hippocampi were placed in 2.5% trypsin for 5 minutes at 37°C. After manual trituration for 1 minute, 5mL of DMEM-FCS-AA (Dulbeco's Modified Eagle Medium with 10% (v/v) fetal calf serum and 1% (v/v) Antibiotic / Antimycotic, Gibco) was added and the suspension spun down at 1,000g for 5 minutes. The supernatant and dead cells were removed and the pellet resuspended in 10mL DMEM-FCS-AA. Two separate 10µL samples of the suspension were placed on a haemocytometer and counted and the average used to determine total cell number.

Cells were plated at 150,000 cells per well in a 24-well plate on 1mm thick glass coverslips coated with 50µg/mL Poly-L-Lysiene. After being left for 18 hours, a complete media change was performed to Neurobasal Media with 2% (v/v) B27 (Invitrogen) supplement and 0.5 M GlutaMAX™ (Gibco). A half media change was performed at 4 and 7 days *in vitro* (DIV) before drug treatment began on day 8. All drug doses were given in a half media change and took place at 8, 11 and 14 DIV. These time points were chosen based on pilot data performed previously in the lab. For examination of the acute effect of drug a single dose was given on day 15, 8 hours prior to fixing the cells. The control dose was 0.1% DMSO. Cultures were also co-treated with either 50µM indinavir (Sigma-Aldrich) or 100µM or 200µM of an MMP-9 inhibitor (CAS 204140-01-2, Merck) to examine the effect of glucose uptake and MMP activity respectively on

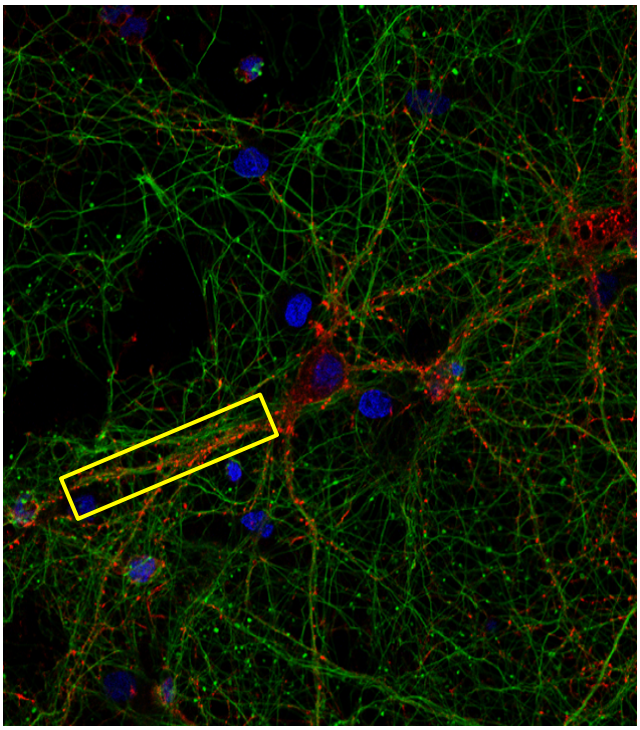
spine growth. Lastly, BDNF (100ng/mL) was used a positive control (Cohen et al. 2008)

3.2.4 Immunohistochemistry and quantification of dendritic spines

Immunocytochemistry and image analysis of spine density and functionality was performed as previously described (Diwakarla et al. 2016a). At 15 DIV, cells were washed with 37°C PBS before being fixed with 4% w/v PFA in PBS for 10 minutes before being blocked and permeabilized with 10% (v/v) normal donkey serum (Sigma-Aldrich) and 0.3% (v/v) Triton-X in PBS. The primary antibodies rabbit anti-β III-tubulin (1:500, Sigma-Aldrich) to stain for neuronal processes and mouse anti-drebrin (1:500, Enzo Life Sciences) to stain for the dendritic spines were applied for 90 minutes at room temperature in PBS. For spine functionality experiments, cells were either incubated with the presynaptic glutamatergic synapse marker rabbit anti-vesicular glutamate transporter 1 (VGLUT1; 1:500; Abcam, Cambridge, MA) or the universal presynaptic marker rabbit anti-synapsin1 (1:500; Abcam). Cells were then incubated for one hour at room temperature with the appropriate fluorescent-conjugated secondary antibodies (1:500; AlexaFluor 488 and AlexaFluor 568; Invitrogen). Cultures were counterstained with DAPI (Merck Millipore, Bedford, MA) and mounted with MOWIOL anti-fade mounting medium (Sigma-Aldrich).

Images were captured using a Zeiss LSM700 inverted confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with a ×63 oil immersion lens. Stubby/mushroom-like spines and filopodia/thin-like spines were identified and counted as previously described (Diwakarla et al. 2016a). Spines staining positive for drebrin, VGLUT1, or synapsin were counted on three individual basal dendrites (primary and secondary) from 10 neurons per culture (3 independent cultures) using Image J 1.49e software (National Institutes of Health, Bethesda, MD). Quantification was performed on 50 µm dendritic segments that were at least 50 µm away from the cell body. All images were captured and analyzed while blinded to the treatments. During imaging, the exposure time and luminescence were fixed and images for all groups within the same batch captured in the same session. Immunostaining where the primary antibodies were omitted was performed to ensure specificity of labeling.

A)



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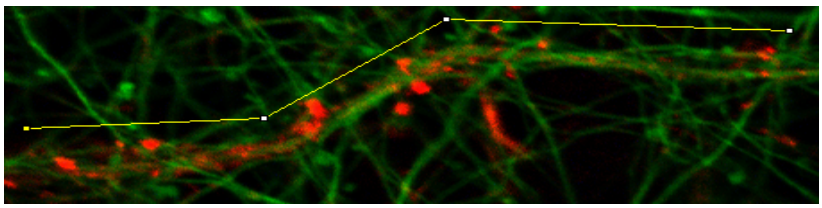


C)

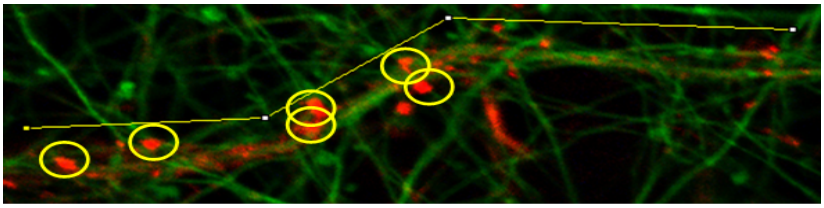


Figure 3-1 Representative image of cultured hippocampal neuron used for quantification of dendritic spines, along with magnified sections showing the measured length

A) A dendrite with minimal crossing branches that could be traced for 50μM and its origin at the pictured cell body were selected for quantification. B) demonstrates the dendritic process chosen for examination and c) shows dendritic spines circled in yellow. A set criterion was used to classify the spines prior to counting with each having to be of sufficient intensity, morphology and also clearly associated with the measured dendrite.

3.2.5 Glucose Uptake

Two treatment protocols were used in the assessment of glucose uptake, similar to dendritic spine assay. To assess the effects of repeat treatment, hippocampal neurons were treated with IRAP inhibitor on 8, 11 and 14 DIV before the assay was performed on day 15. To assess the effects of acute treatment the neurons received a single dose of IRAP inhibitor only in the preincubation buffer prior to the uptake assay on day 15.

To perform the assay, media was removed and replaced with 500µL glucose free NeuroBasal A medium, supplemented with 5mM D-glucose and, in the case of the single treatment, the appropriate dose of IRAP inhibitor dissolved in 100% DMSO (final DMSO concentration 0.1%). Combinations of 40mM potassium to stimulate cells (as the majority of IRAP and GLUT4 are sequestered intracellularly) and 50µM indinavir to block GLUT4 mediated glucose uptake were also added. After 30 minutes 1µCi/mL 2-[1-^{14}C]-Deoxy-D-glucose (PerkinElmer) was added to cultures for an additional 30 minutes. Following this the cultures were washed with 200µL ice-cold PBS three times to stop uptake before the cells were lysed with 0.1% Triton-X-100. Plates were then scraped and 150µL taken and added to 2mL UltimaGold Scintillation buffer (Perkin Elmer) before being counted in a TriCarb 2810 TR Liquid Scintillation Analyser (PerkinElmer)

3.3 Results

3.3.1 Effect of IRAP inhibitors on performance of the spontaneous alternation task

The effect of centrally administered IRAP-Is on memory was assessed using the spontaneous alternation task. In agreement with previous work from our lab (Albiston et al. 2008), a significant treatment effect was observed in rats treated with HFI-419 (Figure 3-2 a) where it improved scores by 10% ($p < 0.01$) compared to those administered DMSO vehicle. This indicates that treated rats were better able to remember their sequence of arm entries and suggests they have improved spatial working. The number of arm entries was also recorded and found not to be different between HFI-419 and vehicle treated rats demonstrating no dug effect on locomotor activity (Figure 3-2 b).

Following this, the novel synthetic inhibitors SJM-164 and VR-006 were assessed in the same task. Both were tested at two doses, 0.1nmol to mirror the effective dose of HFI-419 and also 0.5nmol. As the IC_{50} of SJM-164 is roughly 5-fold lower than HFI-419 it was predicted that this dose should fall within the effective dose range. This simple conversion is made difficult however when considering the substantial variation in metabolic stability between the compounds; even with the same administered dose the concentration that is active at the target site may be substantially different. There was no enhancement of memory noted in response to either inhibitor at either of the tested doses ($p > 0.05$) (Figure 3-3a + Figure 3-4a). While not specifically tested for, of note is that the control animals in the SJM-164 treated cohort appeared to show higher scores than those of the HFI419 group, while the control animals of the VR-006 cohort demonstrated lower scores. No discernable reason could be formulated as to why this may have been the case; animals were handled as with the HFI-419 cohort and the set up of the behavioural testing room identical. Nonetheless, the variation within each group is small suggesting an effect on the entire cohort, rather than increased individual variability.

While the group numbers of these experiments is relatively low ($n = 5 – 6$/group) the confidence interval is small and there is no evidence of a trend towards increased scores with any of the doses tested. Due to the bell shaped dose response, simply increasing the dose is not guaranteed to produce

enhancement. When considering that the selected doses could have fallen on either side of the bell-shaped response curve and when combined with the lengthy process of behavioral testing it was decided that it would be more efficient to focus on the development of the *in vitro* bioassay to screen for which IRAP-Is display cognitive enhancing properties.

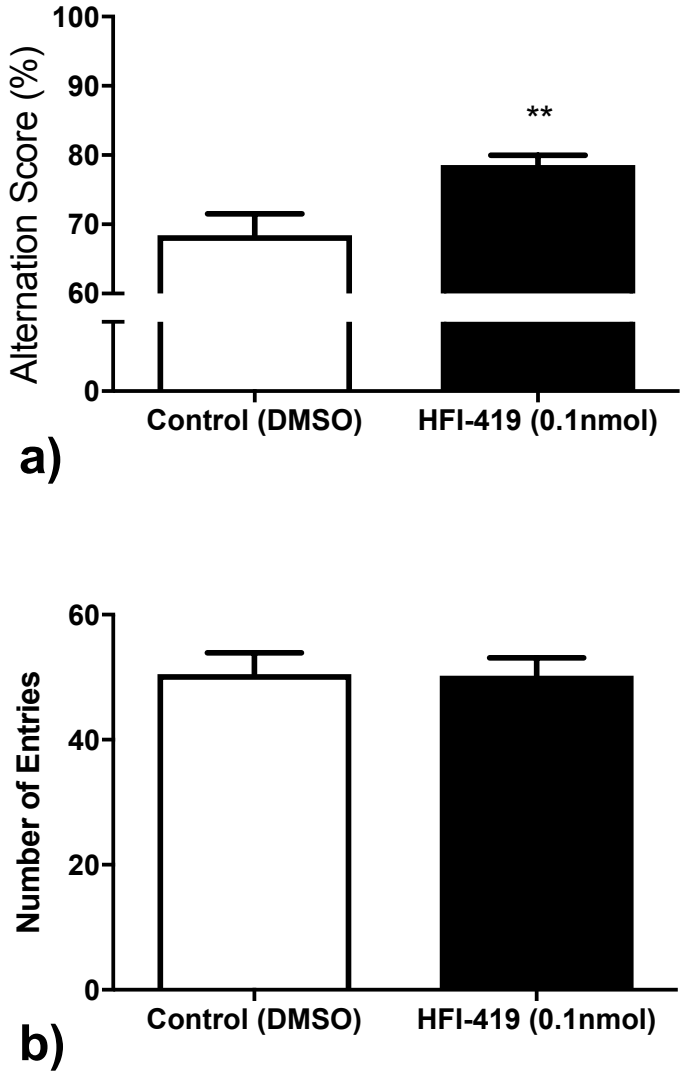


Figure 3-2 Performance on the spontaneous alternation task by rats treated with HFI-419

(a) Rats treated with HFI-419 (0.1nmol) performed significantly better than those treated with control solution (1% DMSO) (p = 0.0096) (b) There was no difference in the number of arm entries between either group (p = 0.95)

*Data represented as mean ± SEM, P value determined by two-tailed unpaired t-test, ** P < 0.01 significantly different from control*

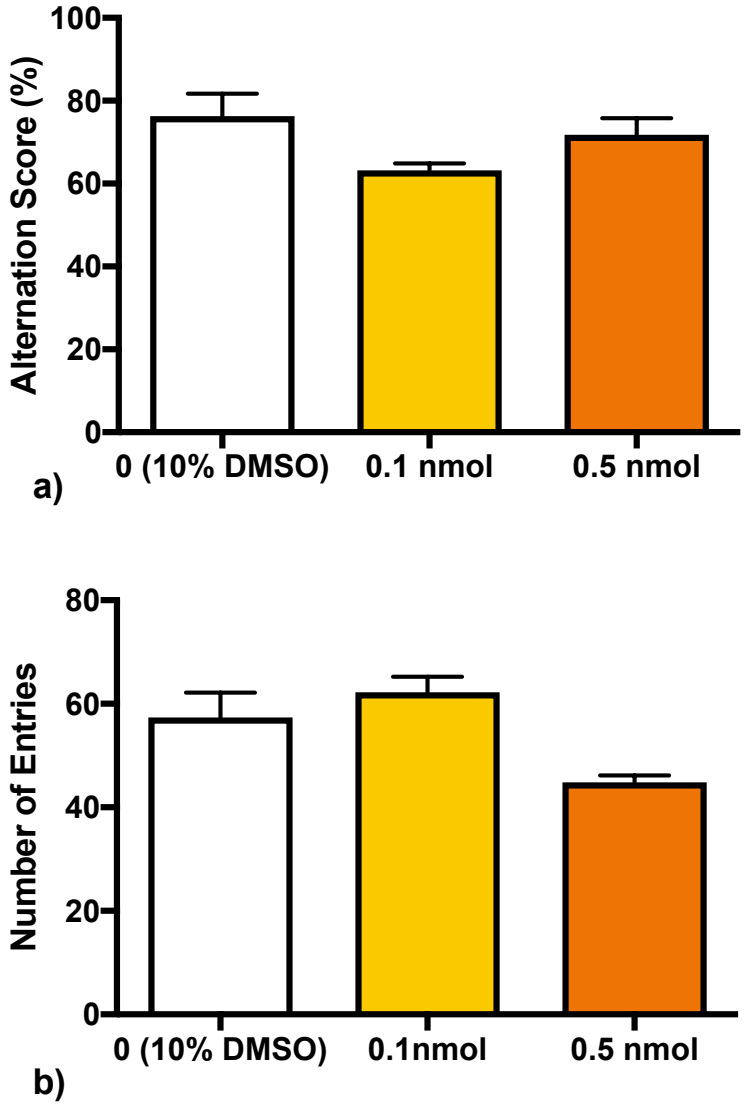


Figure 3-3 Performance on the spontaneous alternation task by rats treated with SJM-164

(a) There was no difference in the performance of rats treated with SJM-164 (either 0.1 or 0.5 nmol) or control solution (1% DMSO) ($p = 0.12$, $F(2,13) = 2.47$) (b) There was no difference in the number of arm entries between either group ($p = 0.16$, $F(2,13) = 1.27$) ($n = 5 - 6$/ group)

Data represented as mean ± SEM, P value determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons, testing for an effect of inhibitor on either alternation score or number of entries

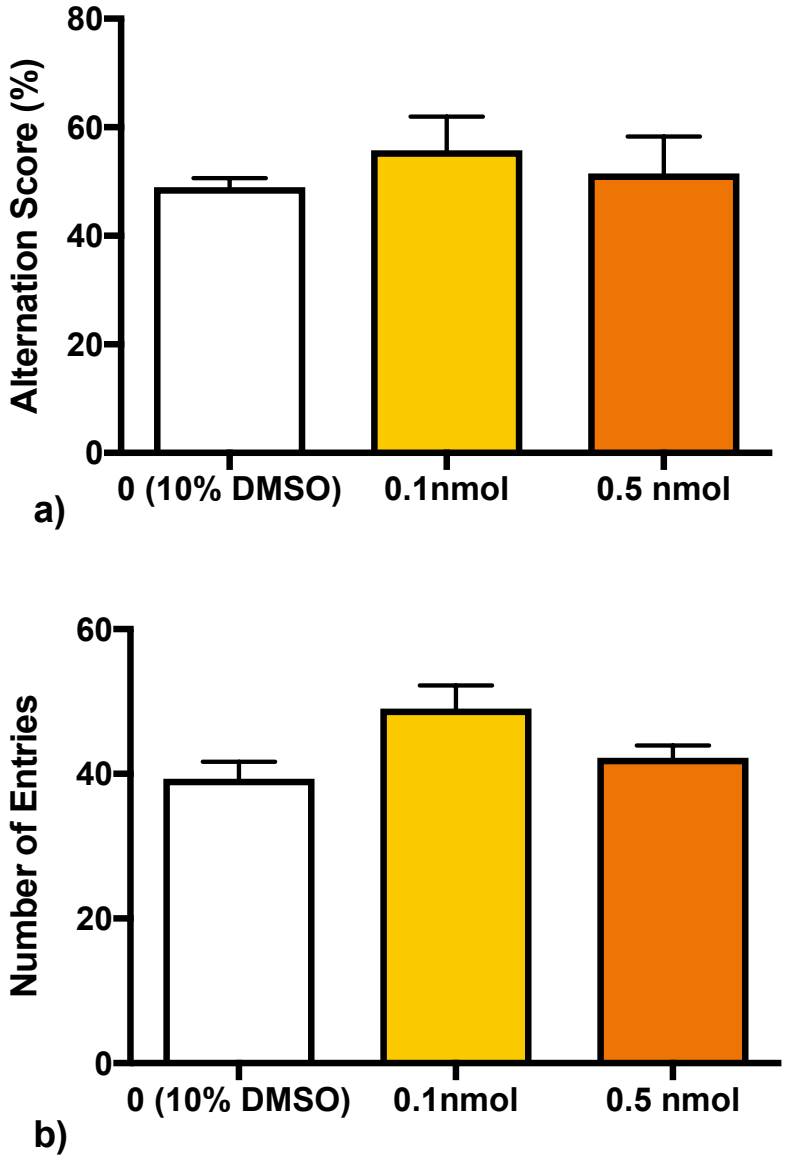


Figure 3-4 Performance on the spontaneous alternation task by rats treated with VR-006

(a) There was no difference in the performance of rats treated with VR-006 (either 0.1 or 0.5 nmol) or control solution (1% DMSO) (p = 0.63, F (2, 13) = 0.47) (b) There was no difference in the number of arm entries between either group (p = 0.07, F (2, 13) = 3.443) (n = 5 - 6/ group)

Data represented as mean ± SEM, P value determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons, testing for an effect of inhibitor on either alternation score or number of entries

3.3.2 *Effect of IRAP inhibition on dendritic spine density in cultured hippocampal neurons*

To address some of the issues regarding *in vivo* screening of novel IRAP-Is, particularly the difficulty in reconciling differences in affinity and bioavailability, the development of an *in vitro* bioassay would allow for preliminary screening of compounds that are capable of enhancing cognition. One possibility is the use of the dendritic spine assay that was developed in collaboration with our partners in Sweden. A major benefit of assessing dendritic spine density is that it directly correlates with cognitive status *in vivo* (Kasai et al. 2010).

When compared to vehicle-treated cells, the spine density of cultured hippocampal neurons was seen to increase in response to three repeat treatments of both HFI-419 (Figure 3-5b) and SJM-164 (Figure 3-6a), however no effect was noted with VR-006 (Figure 3-6b). There was a bell shaped dose response, with peak responses at 1μM and 0.1μM to HFI-419 and SJM-164 respectively, similar to our previous work using other inhibitors (Diwakarla et al. 2016a, Diwakarla et al. 2016b) highlighting that this dose response is an intrinsic property of targeting IRAP. The ability of a single acute dose to promote changes in spine morphology was also examined, however treatment of cultures with a single dose of HFI-419 eight hours prior to fixing was unable to promote increased dendritic spine density (Figure 3-5a). In a separate set of experiments conducted in Sweden, the ability of HFI-419 to enhance dendritic spine density was confirmed (Figure 3-8a). Here the morphology of the dendritic spines was also examined with IRAP-I treatment increasing mushroom spine density (Figure 3-8b) (a morphology associated with the encoding of information and retention of memory), to a similar extent as the well-known cognitive enhancer BDNF (Figure 3-8), however there was no significant effect on the density of thin spines.

While the IRAP-Is may promote the growth of mushroom spines, whether they form part of a functional synapse is also critically important. To determine this, immunohistochemical staining against the presynaptic markers synapsin1 and vesicular glutamate transporter 1 (VGLUT1) was performed on cells

exposed to HFI-419 in an assay co-developed with our collaborators in Sweden. Synapsin1 is required for maintaining vesicles at excitatory synapses (Gitler et al. 2004), while the presence of presence of VGLUT1 demonstrates the ability of the spines to engage in glutamataminergic signaling, a key process in memory formation (Staubli et al. 1994, Riedel et al. 2003). Presence of both of these markers indicates that the dendritic spine has made contact with a functional presynaptic bouton. Compared with the vehicle control, there was no difference in the proportion of drebrin-positive and VGLUT1 (Figure 3-9a) or synapsin-positive (Figure 3-9b) spines, indicating that HFI-419 treatment not only promotes the increased growth of mushroom spines, but that these spines are functional. To expand on this point, as the IRAP-Is increased the spine density, the proportion of spines expressing synapsin and VGLUT1 was also correspondingly higher. Therefor the ratio of synapsin/VGLUT1 positive spines to drebrin stained spines remained the same as the vehicle group. This indicates that IRAP-I treatment results in the same proportion of functional spines as the control group, but an overall greater number of spines.

To begin to investigate the mechanism through which IRAP-Is are able to enhance spine growth, hippocampal cultures were co-treated with HFI-419 and either indinavir to block GLUT4 mediated glucose uptake (Murata et al. 2002) or an MMP9 inhibitor. Neither dose of the MMP9 inhibitor (100μM or 200 μM) affected dendritic spine density, however indinavir (50μM) completely abolished the increase in spine growth (Figure 3-10) indicating the importance of glucose uptake through GLUT4 in driving increased spine growth. Of note, while indinavir is an aspartyl protease inhibitor and typically thought of as an antiviral agent, it has also been demonstrated to block GLUT4 mediated glucose uptake (Murata et al. 2002), likely by binding to a zHHe peptide sequence (Hertel et al. 2004).

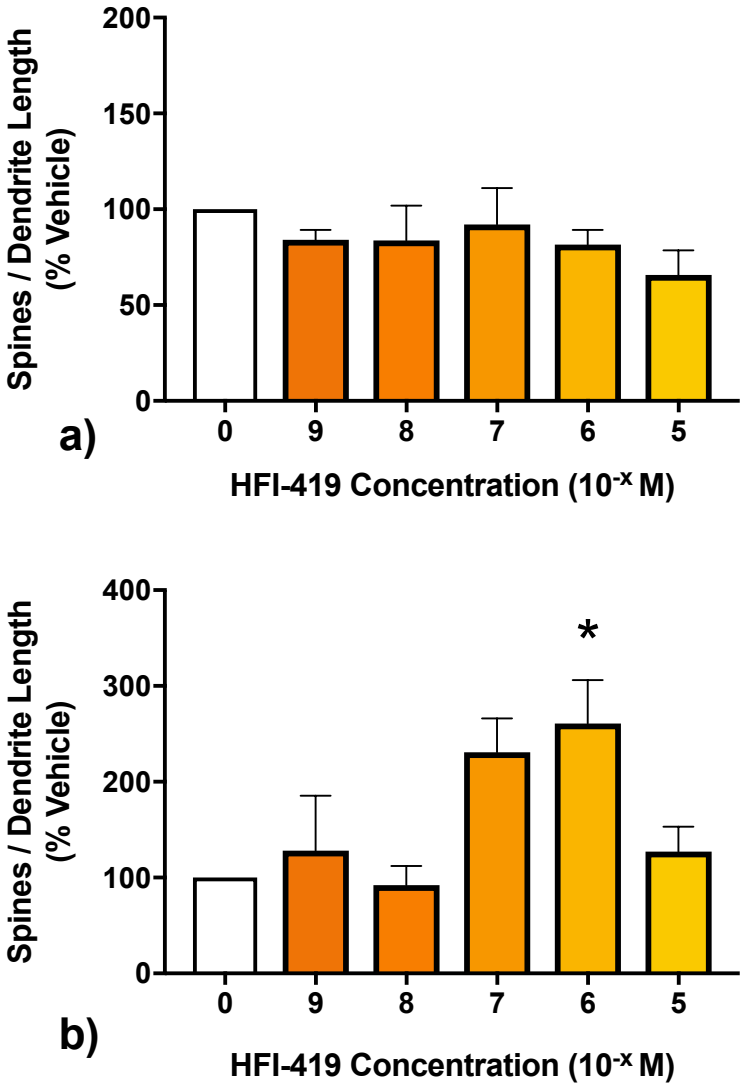


Figure 3-5 Spine density in cultured hippocampal neurons treated with either a single or three repeat doses of HFI-419

The $^{-x}$ refers to the dose on the x-axis a) *While there was no difference in the density of dendritic spines following a single treatment of HFI-419 ($p = 0.5361$, $F(5,24) = 0.8376$), b) three treatments produced a bell shaped dose response ($p = 0.0132$, $F(5,18) = 0.7513$) dendritic spine density with a significant effect with the 10^{-6}M dose.*

*Data presented as mean ± SEM, n = 4 - 5, p value determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons, testing for an effect of inhibitor on the density of dendritic spines, * $p < 0.05$ compared to control*

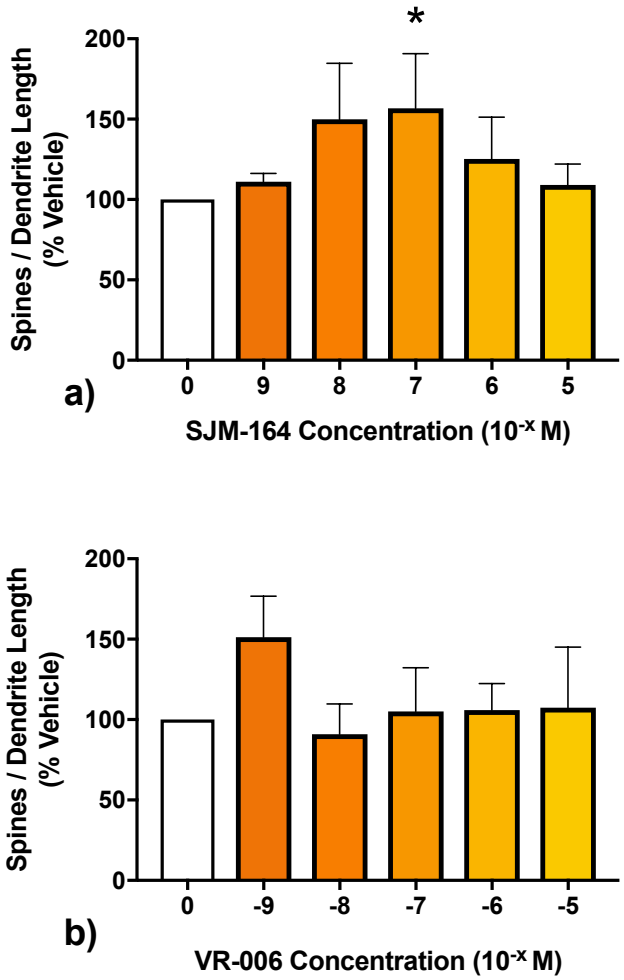
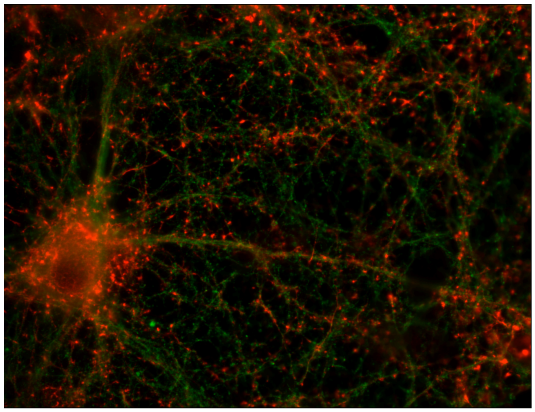


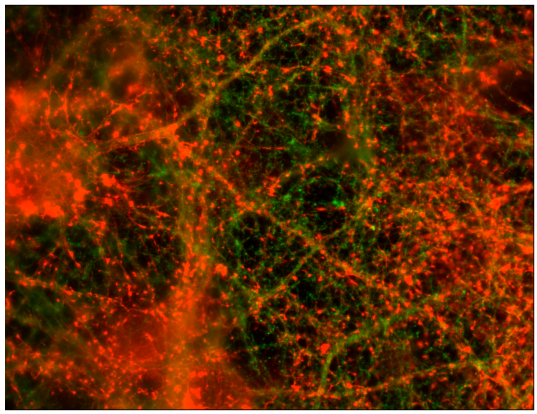
Figure 3-6 Spine density in cultured hippocampal neurons treated with varying doses of either SJM-164 or VR-006

The $^{-x}$ refers to the dose on the x-axis *a) Treatment with the novel IRAP inhibitor SJM-164 produced the characteristic bell-shape dose response in dendritic spine growth, with the most effective dose in this case being 10^{-7}M ($p = 0.036$, $F(5,18) = 0.5134$) b) VR-006, a structurally distinct IRAP inhibitor, was unable to promote dendritic spine growth at any dose however ($p = 0.5816$, $F(5,18) = 1.621$)*

*Data presented as mean ± SEM, n = 4 – 5, p value determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons, testing for an effect of inhibitor on the density of dendritic spines, * $p < 0.05$ compared to control*



a) Control



b) HFI-419

Figure 3-7 Cultured hippocampal neurons treated with either vehicle control (0.1% DMSO) or 1µM HFI-419

Drebrin, highly concentrated in dendritic spines, is visualized in red while the green highlights tublin, a cytoskeleton protein used to determine the path of the neuronal processes. a) Hippocampal neurons treated with the vehicle alone (0.1% DMSO) show some dendritic spine growth, however b) this is greatly enhanced in those cultures treated with 1µM HFI-419. Exposure time and luminescence were fixed for both images.

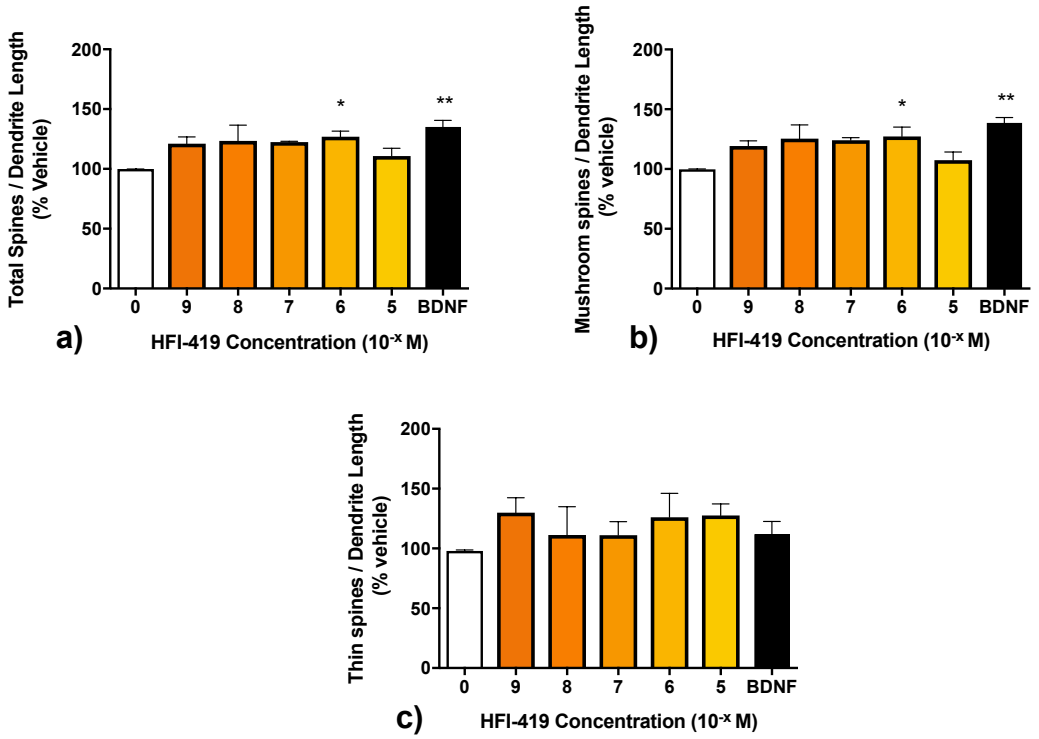


Figure 3-8 Comparison of the types of spine growth that is promoted in response to treatment with the IRAP inhibitor HFI-419

a) In a separate set of experiments, the ability of HFI-419 to enhance dendritic spine density in a bell shaped dose was confirmed ($p = 0.0412$, $F(6,14) = 0.9091$) b) The proportion of mushroom spines was increased in cultures that were treated with 10^{-6}M HFI-419 and the positive control BDNF ($p = 0.0134$, $F(6, 14) = 0.4216$) and a slight bell shaped dose is noted. In contrast, c) the proportion of thin spines was not altered by any HFI-419 dose or the BDNF positive control ($p = 0.6835$, $F(6,14) = 0.6351$)

*Data represented as mean ± SEM, p valued determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons, testing for an effect of inhibitor on dendritic spine density, * $p < 0.05$ and ** $p < 0.01$ compared to control*

Experiments performed by Shanti Diwakarla in co-developed assay

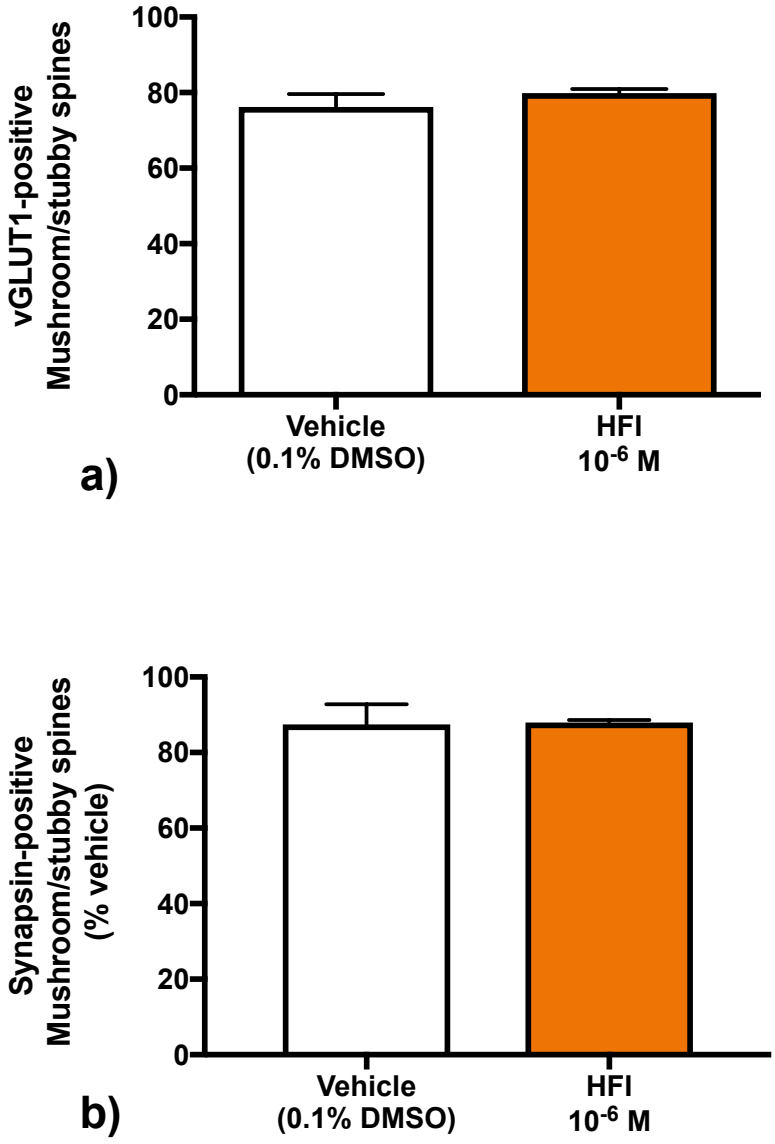


Figure 3-9 Comparison of the proportion of synapsin and VGLUT1 in mushroom spines in dendritic spines exposed to 1µM HFI-419

Cultures treated with 1µM HFI-419 showed no significant difference in the percentage of drebrin-positive and a) vGLUT-1 or b) synapsin-postive spines indicating a similar proportion of functional spines between cultures.

Data presented as mean ± SEM, n = 3, p value determined by t-test testing for an effect of inhibitor on the proportion of spines colocalising synapsin and VGLUT1 with drebrin

Experiments performed by Shanti Diwakarla in co-developed assay

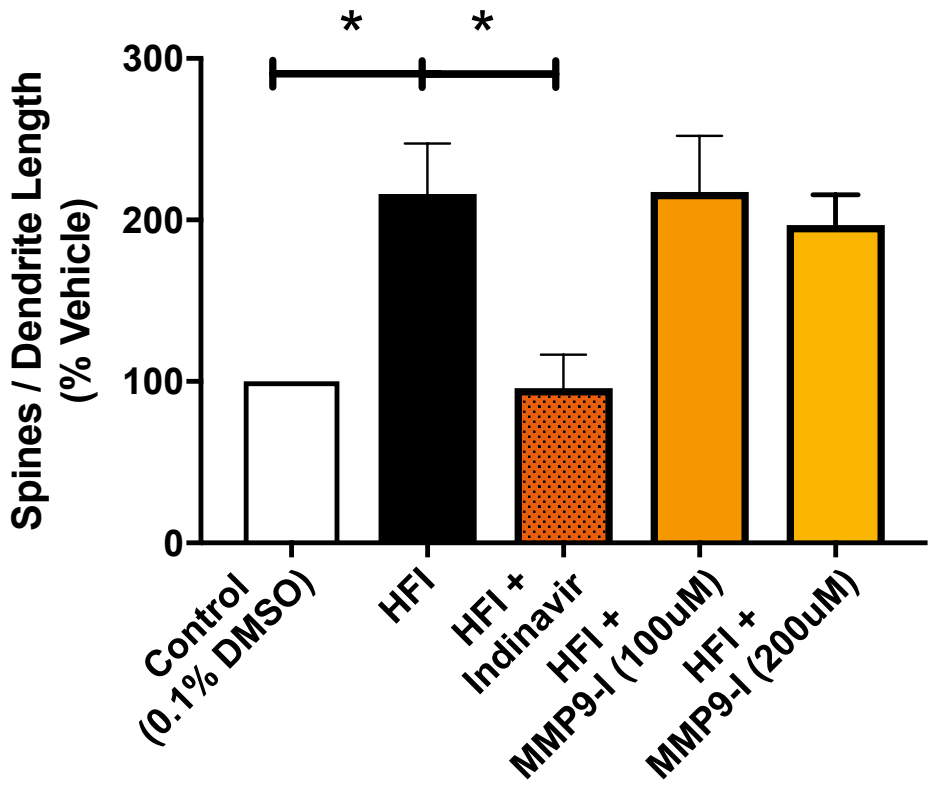


Figure 3-10 Cultured hippocampal neurons treated with either vehicle (0.1% DMSO) or HFI-419 (1μM) and co-treated with either Indinavir or MMP9 inhibitor

Hippocampal cultures treated with 1μM HFI-419 showed increased dendritic spine density (p = 0.014), however this increase was abolished by the addition of 50μM Indinavir (p = 0.01), which blocks GLUT4 mediated glucose uptake. Dendritic spine density was unaffected by the addition of the MMP9 inhibitor at two doses, 100μM and 200μM.

*Data represented as mean ± SEM, n = 4, p valued determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons against HFI-alone, testing for an effect of inhibitor on dendritic spine density, * p < 0.05 compared to HFI-alone*

3.3.3 *Effect of IRAP inhibitors on glucose uptake in cultured hippocampal neurons*

The attenuation of increased dendritic spine density following IRAP inhibition in the presence of indinavir indicated that GLUT4 (and thus glucose uptake) plays an important role in enhancing spine formation. The ability to measure glucose uptake in hippocampal cell cultures would provide an ideal bioassay to screen for IRAP-Is with potential cognitive enhancing properties. Of interest was whether treatment with IRAP-Is fundamentally altered the ability of the neurons to take up glucose, or whether the increase was only an acute effect. Cultures that were only treated with a single dose of HFI-419 in the preincubation buffer produced a bell-shaped dose response with the same effective dose as seen in the spine assay (1μM) (Figure 3-11a), however, cells treated similar to those in the spine assay (three repeat treatments on days 8, 11 and 14 but not in the preincubation buffer) did not appear to alter glucose uptake (Figure 3-11b)

Based on this, testing with the novel IRAP-Is was performed using the acute dosing scheme (a single dose in the pre-incubation buffer). Here, SJM-164 also exhibited a bell shaped dose response; with a peak effective dose (0.1μM) the same as the spine assay (Figure 3-12a). Also, similar to the spine assay, VR-006 did not cause an increase in glucose uptake in hippocampal neurons (Figure 3-12b).

In order to confirm that the increase in glucose uptake was mediated by GLUT4, the cultures were also co-treated with indinavir, which resulted in complete abolishment of the increase in glucose uptake (Figure 3-13). Previous literature has suggested that for glucose uptake to be increased, cells must be stimulated in order to promote IRAP and GLUT4 translocation to the plasma membrane, however no further increase in glucose uptake was noted here with the addition of 40mM potassium (Figure 3-13). It is possible that this was due to the hippocampal cells being in culture and thus in a 'stimulated' state already.

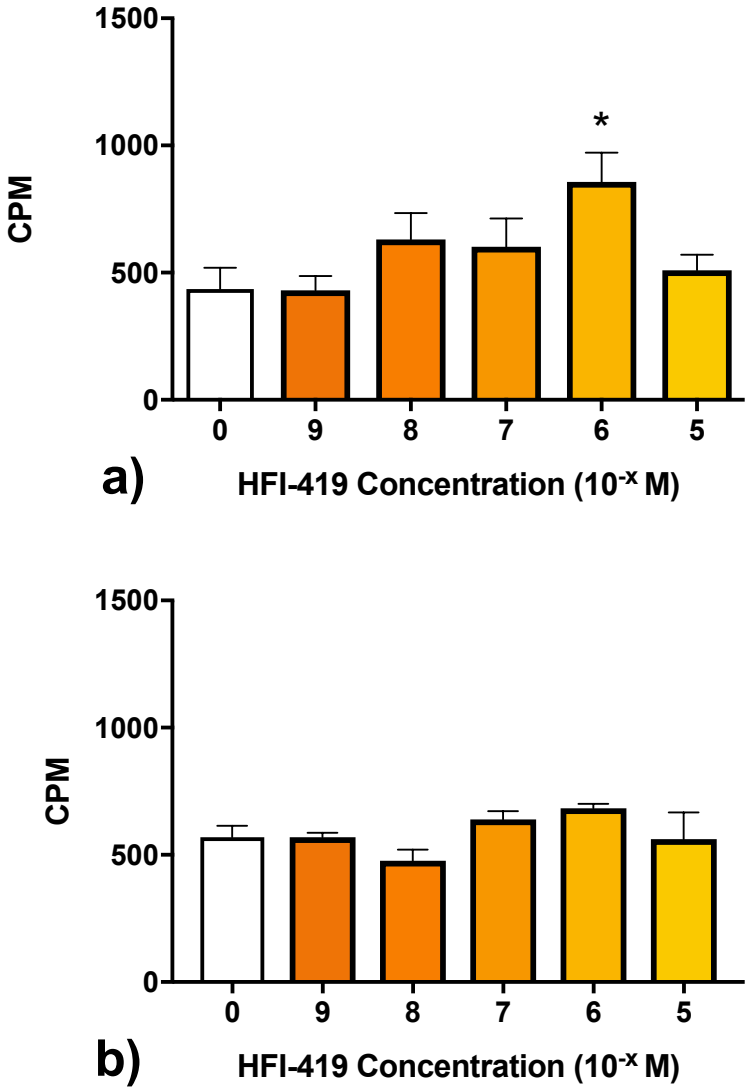


Figure 3-11 Glucose uptake in cultured hippocampal neurons treated with either a single acute dose of HFI-419 or three repeat treatments across one week

Glucose uptake in cultured hippocampal neurons treated with a single acute dose of HFI-419 demonstrated a bell shaped dose response ($p = 0.036$, $F(5,23) = 0.4446$) with the most effective dose being 10^{-6}M while the repeat treatment did not affect glucose uptake ($p = 0.1787$, $F(5,12) = 1.341$)

*Data represented as mean ± SEM, n = 3 -5, p valued determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons, testing for an effect of inhibitor on glucose uptake, * $p < 0.05$ compared to control*

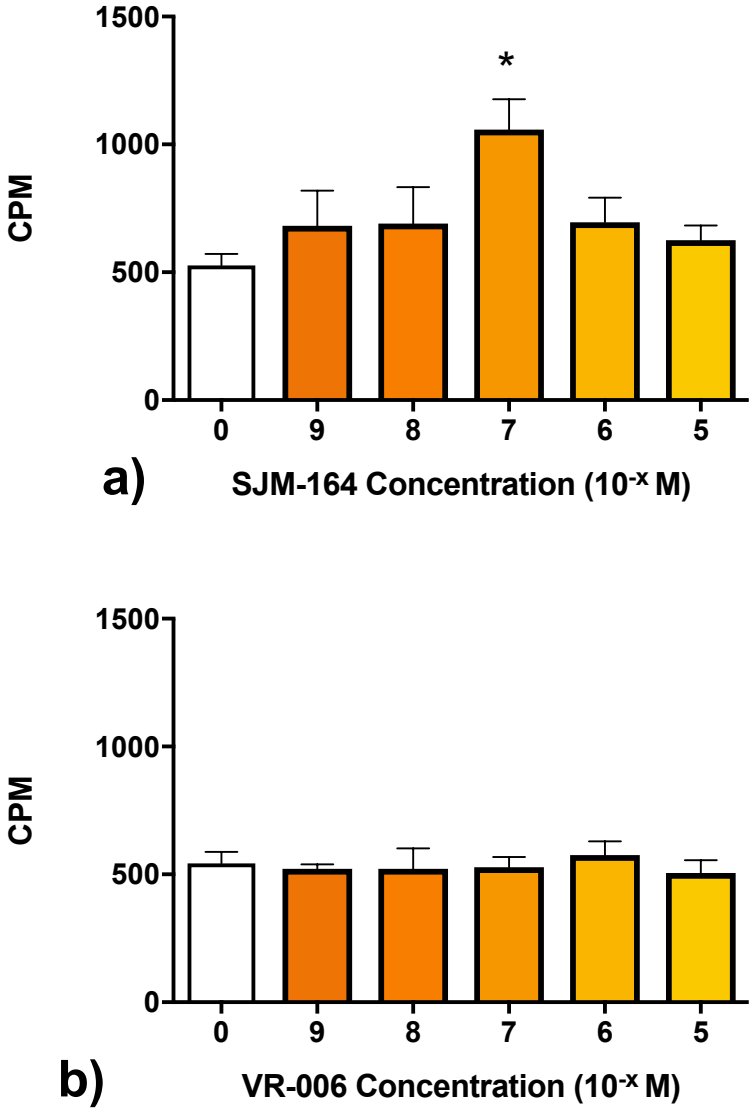


Figure 3-12 Glucose uptake in cultured hippocampal neurons treated with either SJM-164 or VR-006

Glucose uptake in cultured hippocampal neurons demonstrated a bell shaped dose response to a) SJM-164 ($p = 0.043$, $F(5,18) = 0.7259$) with the most effective dose being 10^{-7}M. However, b) VR-006 was unable to promote an increase in glucose uptake at any dose ($p = 0.9453$, $F(5,12) = 0.2473$)

*Data represented as mean ± SEM, n = 3 -5, p valued determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons, testing for an effect of inhibitor on glucose uptake, * $p < 0.05$ compared to control*

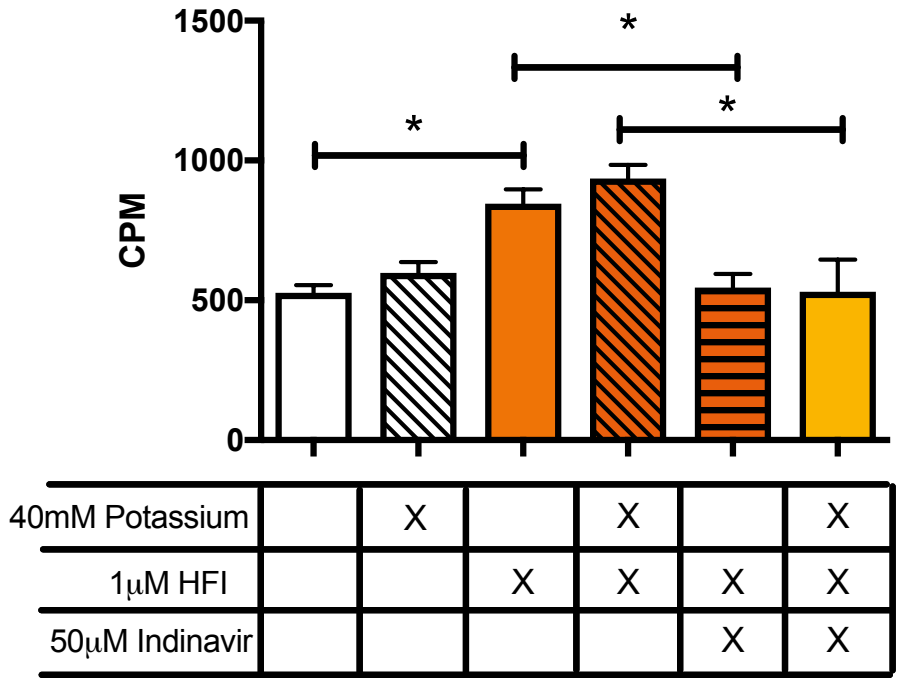


Figure 3-13 Glucose uptake in cultured neurons treated with HFI-419 under various stimulation conditions

Hippocampal neuronal cultures treated with 40mM K^+ did not exhibit enhanced glucose uptake compared to control (p = 0.99). Treatment with 1μM HFI-419 produced the previously observed roughly 2-fold increase in glucose uptake compared to control (white column) (p = 0.027) and the addition of K^+ did not appear to produce a further increase (p = 0.99). The addition of 50μM Indinavir abolished the enhanced glucose uptake promoted by HFI-419 treatment alone (0.04) or in the presence of K^+ (p = 0.0031).

*Data represented as mean ± SEM, n = 5, * p < 0.05, p valued determined by 1-way ANOVA followed by a post-hoc Dunnet's multiple comparisons*

3.4 Discussion

3.4.1 Validation of the cognitive enhancing properties of HFI-419

While the present work did not demonstrate cognitive enhancement resulting from treatment with SJM-164 or VR-006, it did replicate previous work from our lab (Albiston et al. 2008) and supported the ability of HFI-419 to enhance cognition in the spontaneous alternation task. While the novel IRAP-Is were unable to promote increased cognition here, there are several factors that could have affected this.

The most likely reason is due to the aforementioned narrow bell shaped dose response, a feature common to the IRAP-Is (Benoist et al. 2011, Diwakarla et al. 2016) and shared by many nootropics (Pepeu et al. 1989, Moriguchi et al. 2013, Stoiljkovic et al. 2015). The 0.1nmol dose used here was chosen to mimic the effective dose of HFI-419 and the 0.5nmol to account for the 5-fold lower affinity of SJM-164. However as discussed, this simple conversion is complicated by the vastly different bioavailability of the compounds. This means that although the administered dose might be the same, it could result in an unpredicted dose being active at the target site (IRAP in the hippocampus in this instance); thus the doses chosen here may have been too high or low to produce an enhancement of cognition.

Another possibility that must be considered is that not all IRAP-Is are able to promote cognitive enhancement. Previously it has been demonstrated that AngIV and LVV-H7 (another peptide inhibitor of IRAP) produce differential effects on hippocampal acetylcholine levels (De Bundel et al. 2010) and that only some peptidomimetics induce increased spine growth (Diwakarla et al. 2016). It is therefore conceivable that even though all of the IRAP-Is used here are capable of inhibiting IRAP with micromolar potency, they may produce differential effects on cognition. Considering these points and taking account that there was no current method of determining whether to increase or decrease the doses, as well as the lengthy time and budget considerations for behavioural testing, it was decided to discontinue the memory testing in the confines of the PhD. Instead, efforts were focused on the determination of the

mechanism of action and development of a *in vitro* bioassay to screen novel IRAP-Is with potential cognitive enhancing effects.

3.4.2 IRAP inhibition enhances dendritic spine density

Previously, our collaborators demonstrated the efficacy of the macrocyclic and aryl sulfonamide derived IRAP-Is at enhancing dendritic spine density in primary hippocampal cultures (Diwakarla et al. 2016a, Diwakarla et al. 2016b), however there has been no behavioural work performed with these inhibitors to confirm that these effects translate to enhanced cognition *in vivo*. This study extends on the previous work by demonstrating that the synthetic IRAP-I, HFI-419, is able to enhance cognition as seen in the spontaneous alternation task, and also increase dendritic spine density in cultured hippocampal neurons. This validates the dendritic spine assay as a potential *in vitro* screen for IRAP-Is with possible capacity for cognitive enhancement.

This work demonstrated that both HFI-419 and SJM-164 enhanced dendritic spine density in a bell-shaped dose-response manner, indicating a class effect of the IRAP-Is. The bell shaped dose response on dendritic spine density in response to IRAP-I treatment was notable. As mentioned, it is seen here with the benzopyran inhibitors, by our collaborators work using the macrocyclic inhibitors (Diwakarla et al. 2016a) as well as earlier experiments with angiotensin IV derivatives (Benoist et al. 2011) and highlights that it is a key feature of the IRAP-Is across all of the inhibitor families. This suggests that the inhibitors are likely to display a similar response *in vivo* when attempting to enhance cognition. These results show the need to screen IRAP-Is using an *in vitro* assay before progressing to behavioral work. As highlighted here, the narrow bell shape makes it difficult to determine the appropriate dose at which to treat animals and the lack of efficacy of VR-006 at enhancing spine growth shows that this may have been in vain.

Looking to the specific type of spine growth, IRAP-I treatment promoted an increase the proportion of mushroom spines. These spines are associated with the encoding of information and improved memory (Beltran-Campos et al. 2011) and supports the cognitive enhancing properties of IRAP-I treatment. Further, the proportion of synapsin1 and VGLUT1 were the same between cells

treated with vehicle and those with HFI-419, indicating that these cells have an increased capacity for excitatory synaptic input, a process important for memory formation. Specifically, synapsin1 is required for maintaining vesicles at excitatory synapses (Gitler et al. 2004) and VGLUT1 demonstrates the ability of the spines to engage in glutamate signaling (Staubli et al. 1994, Riedel et al. 2003). Presence of both of these markers indicates that the dendritic spine has made contact with a functional presynaptic bouton and in turn capable of participating in memory formation. Taken together this work demonstrates that IRAP-I treatment is able to make alterations in neuronal structure to promote lasting changes to memory and learning.

The hippocampal cell culture assay also allows for a mechanism of action of IRAP inhibition to be investigated. Several lines of evidence have shown the close relationship between IRAP and GLUT4 (Jiang et al. 2001, Albiston et al. 2008, Fernando et al. 2008) and unpublished work from our collaborators suggests an interaction with the MMP/TIMP system (Lee, unpublished). Here, blockade of GLUT4 mediated glucose uptake abolished spine growth while blockade of MMP9 had no effect, in turn leading to a further investigation of the ability of the IRAP-Is to enhance glucose uptake.

3.4.3 IRAP inhibition increases glucose uptake in hippocampal neuronal cultures

As indinavir completely abolished the IRAP-I mediated increase in spine density, the direct effect of the IRAP-Is on glucose uptake in the hippocampal cell cultures was assessed. Notably, in the glucose uptake assay, both HFI-419 and SJM-164 demonstrated bell-shaped dose responses with the peak efficacy matching that from the spine assay, while VR-006 was unable to promote an increase in glucose uptake. The abolishment of spine growth in response to indinavir treatment suggests that IRAP-I treatment is causing an increase in dendritic spine density through an increase in GLUT4 mediated glucose uptake. Previously, stimulation with potassium has been required in order to see an effect of the IRAP-Is on glucose uptake (Albiston et al, 2008), however, here no additional glucose uptake was noted. This may be due to the fact the hippocampal neurons are already in a 'stimulated' state when in culture as they likely do not have the same regulated environment they would have *in vivo*.

In this study VR-006 treatment was unable to promote either increased dendritic spine growth or glucose uptake. Speculating as to the possible reasons that VR-006 did not produce an effect is difficult however, as the precise mechanism by which the IRAP-Is are able to increase glucose uptake is currently unknown. It has been suggested that binding of the inhibitors interferes with IRAP and GLUT4 endosome recycling (Fernando et al. 2008), thus prolonging their time at the plasma membrane. It is possible that while VR-006 is able to bind to the active site of IRAP and inhibit its catalytic activity, its unique structure does not interfere with the recycling pathway, thus GLUT4 is not prolonged at the membrane, there is no increase in glucose uptake and therefore no subsequent increase in dendritic spine density or cognition.

3.4.4 Translation to in vivo work

Of interest at this point is that in order for increased spine growth to occur, three repeat doses of inhibitor were required, while memory enhancement and glucose uptake are both seen in response to a single acute doses. Indeed, acute glucose loading is well reported to acutely enhance memory both in animal models (Kopf et al. 2001) and in humans (Smith et al. 2011, Macpherson et al. 2015), particularly in hippocampal dependent tasks (Glenn et al. 2014). This would suggest that the IRAP-Is are enhancing glucose uptake and memory through an acute mechanism and the dendritic spine density through another and that these may not necessarily be linked. However, co-treatment of the cultures with indinavir abolished the increase in dendritic spine growth, demonstrating the crucial role GLUT4 facilitated glucose uptake plays in mediating the response to the IRAP-Is. Further there are likely two potential mechanisms through which increased glucose uptake may promote an acute increase in cognition and then later drive an increase in dendritic spine density.

The IRAP-Is have been noted to increase cholinergic transmission (Lee et al. 2001) and ameliorate deficits resulting from AChR antagonism (Olson et al. 2004). In turn, the memory enhancing effects of glucose have been suggested to result from a central cholinergic mechanism, with glucose both enhancing ACh output (Ragozzino et al. 1996) and cholinergic antagonists such as atropine blocking the memory enhancing effects of glucose (Kopf et al. 1994). In turn,

activation of AChRs has been shown to induce plasticity, both enhancing LTP (Dennis et al. 2016) and altering dendritic structure (Oda et al. 2014), particularly in hippocampal neurons. Another potential mechanism is through an increase in the activity of mammalian target of rapamycin (mTOR), activation of which triggers a cascade resulting increased protein synthesis, crucial dendritic spine growth (Dash et al. 2006). Indeed inhibition of mTOR attenuates cognitive enhancing effects of glucose (Dash et al. 2006).

Notably, both of these mechanisms demonstrate that the acute administration of IRAP-Is results in increased neuronal glucose uptake and that this is able to trigger further down-stream pathways that go on to alter neuronal architecture and promote lasting changes to cognition. It is possible that the effects of the IRAP-Is are two-fold, acting acutely to drive glucose uptake into neurons and increasing cholinergic release (Ragozzino et al. 1998), which improves performance on working memory tasks such as the spontaneous alternation task, while also activating downstream pathways such as the mTOR cascade to promote long-term changes to dendritic architecture. Further investigation of these mechanisms should form the basis of further investigation.

In terms of AD, the GLUT4 mediated glucose uptake by the IRAP-Is could also prove promising. The AD brain features a down-regulation of GLUT1 and GLUT3 that was correlated to increased tau hyperphosphorylation (Liu et al. 2008). The authors speculated that decreased glucose uptake led to increased oxidative stress within the cell, which in turn increased tau phosphorylation and ultimately suggested that increasing glucose uptake could prove to be a valuable therapeutic option. While the study did not examine GLUT4, the premise remains the same. Increasing the availability of glucose inside the cell (through IRAP-I mediated GLUT4 glucose uptake) could reduce oxidative stress and lead to a reduction in tau phosphorylation and overall less pathological damage.

One caveat to discuss is previous *in vivo* work regarding glucose uptake and performance on memory tasks. During tests such as the spontaneous alternation task, as glucose is taken up into cells there is a decrease in glucose levels in the extracellular fluid (McNay et al. 2000), however administration of IRAP-Is does not induce a further decrease (De Bundel et al. 2009), indicating

that it would not appear to stimulating uptake. This is at odds with the current work that demonstrates increased uptake into hippocampal neurons. However, as the authors note, this does not preclude increased neuronal uptake, for example during periods of increased neuronal glucose uptake astrocytes may shift towards lactate as a source of fuel, reducing their glucose consumption rates and thus overall the extracellular pool of glucose would remain constant (Simpson et al. 2007).

Overall, there are two important inferences that can be made about the IRAP-Is from this chapter. First, as the IRAP-Is are likely to mediating their increase in cognition and dendritic spines through an increase in glucose uptake this puts them at an ideal place to act as a cognitive enhancing agent across a broad range of disease states. While a therapeutic that targets a disease specific process, such as amyloid in AD, if the IRAP-Is are able to enhance cognition through a separate mechanism this allows them improve cognition in any disease that features impaired memory as a symptom. Further, age-related cognitive decline is also common and the IRAP-Is could serve as a therapy in those cases.

Secondly, assessment of glucose uptake can serve as a sensitive bioassay to screen for IRAP-Is with potential cognitive enhancing properties. Here it was noted that inhibitors display similar dose response curves on both glucose uptake and dendritic spine growth, suggesting a common mechanism that is confirmed by the abolishment of both by indinavir. Further, in the case of HFI-419 these assays were validated following demonstration of its enhancement of cognition *in vivo.*

3.5 Conclusion

Previous studies have demonstrated the ability of various IRAP-Is to enhance cognition and dendritic spine density separately, however this is the first work to show this effect with the same inhibitor. Here, HFI-419 was able to enhance cognition in the spontaneous alternation task as well as increase dendritic spine density in cultured hippocampal neurons. Given that these spines also expressed synapsin and VGLUT1 lends further support to the ability of the IRAP-Is too make functional changes to synaptic architecture that

promotes increased cognitive capacity. The critical role of glucose uptake through GLUT4 was revealed by the abolishment of enhanced spine growth in the presence of indinavir. This provided a mechanism by which the IRAP-Is were able to enhance cognition and importantly allowed a sensitive *in vitro* bioassay to be developed by which inhibitors could be screened for their cognitive enhancing potential through the use of the glucose uptake assay.

In turn, the need for the assay was highlighted by the initial work of the chapter attempting to demonstrate the ability of the novel synthetic IRAP-Is, SJM-164 and VR-006, to enhance performance on memory tasks, where, despite both compounds demonstrating micromolar affinity for IRAP, neither was capable of enhancing cognition. In the *in vitro* assays however, only SJM-164 was able to both enhance dendritic spine density and glucose uptake. While the precise reason for the lack of efficacy of VR-006 is unclear, it emphasizes that pharmacokinetic analysis is not a sufficient predictor of cognitive enhancing capacity. When considering the tight bell shaped dose response curve that the inhibitors display, factors such as bioavailability and stability make it difficult to determine an effective dose based on kinetics alone. The glucose uptake assay therefore offers a screen that can determine promising compounds without interference from these factors that would make behavioral memory testing difficult.

Now that the likely mechanism mediating cognitive enhancement by the IRAP-Is has been determined, more rapid screening of novel compounds can be performed, particularly to identify those compounds with more favorable characteristics such as improved blood brain barrier permeability. There is also interest as to the efficacy of the inhibitors in disease states where the IRAP-Is may prove to be particularly beneficial. Alzheimer's disease is notable in this case given it features a reduction in cognitive capacity, spine density and glucose uptake, all metrics that IRAP-I treatment has shown to positively affect in this study. As such, this forms the basis of the following chapter, which sought to investigate the efficacy of inhibiting IRAP activity as a potential therapeutic in the treatment of AD.

Chapter 4

Evaluation of the 5xFAD Alzheimer's mouse as a suitable model to assess the therapeutic potential of the IRAP inhibitors

Part 1

4.1 Introduction

With the repeated success of the IRAP-Is at enhancing memory in both normal (Albiston et al. 2008) and cognitively impaired (Wright et al. 1996) rats, there has been interest as to whether they could form an effective therapeutic agent in the treatment of Alzheimer's disease (AD), a progressive neurodegenerative disorder currently affecting approximately 353,000 people in Australia (Heuvel et al. 2012). The disease features not only cognitive impairment as a symptom, but also a reduction in dendritic spine density (Baloyannis 2015), as well as reduced cerebral glucose uptake (Mosconi et al. 2008) as a possible underlying pathological cause, ideally placing inhibition of IRAP as a potential therapeutic option.

In order to study the potential effects of IRAP inhibition on Alzheimer's pathology a suitable animal model is required. While rats would be preferable given they were used in the previous chapters, there are few rat-based models of AD. These include AD induced via central administration of streptozotocin (Grünblatt et al. 2004, Grünblatt et al. 2007) or infusion of amyloid β (Frautschy et al. 1996), however these models only replicate the human condition in a very limited fashion and as such are not widely used within the literature. Transgenic rat models do exist (Galeano et al. 2014), however there is conflicting evidence as to how reliably these express even the basic components of the disease, such as amyloid plaques (Do Carmo et al. 2013). As such it was decided to utilise a mouse model of the disease, as these have far greater use and validation within the literature. In particular, the 5xFAD model was chosen due to the rapid onset of pathology and symptoms, making it an ideal candidate for use with the timeframe of a PhD candidature.

Following preliminary work from the previous PhD student in the lab, it was hypothesised that modulation of IRAP activity could ameliorate plaque pathology and potentially serve as a therapeutic in the treatment of the disease. Furthermore, plaque pathology was initially chosen as it forms the primary endpoint by which many potential therapeutic agents are assessed, thus it would

provide a comparison of the efficacy of the IRAP-Is against current treatments. Further, hypometabolism has been suggested to exacerbate plaque pathology (Galeano et al. 2014), presenting a possible mechanism by which IRAP-I treatment could prove to be effective. As this work formed part of a proof-of-concept, the initial aim of this was to determine whether knockout of IRAP in the 5xFAD mouse could ameliorate the plaque pathology associated with AD.

4.2 Methods

4.2.1 5xFAD mouse breeding and genotyping

All research conducted in this study was approved by the Monash University Animal Welfare Committee (MUAWC) under the Monash Animal Research Platform (MARP) 2011/142 application and performed according to the National Health and Medical Research Council of Australia "Code of practice for the care and use of animals for scientific purposes"

The mice used in this study were the transgenic (APPSwFILon, PSEN1*M146L*L286V) 6799Vas/MmJax model (5xFAD) on a mixed B6/SJL background, strain number 006554, with the original breeding pairs purchased from Jax® Mice & Services (The Jackson Laboratory, Bay Harbour, USA). To obtain hemizygous and wild type offspring, male hemizygous mice were mated with female wild type littermates. For breeding purposes, transgenic males were housed with one wild type female, and males kept with females continuously to maintain breeder output. Pups were weaned at 18 - 21 days postnatal and group housed by sex up to 5 per cage.

Genotyping to confirm the presence of the transgene was performed on DNA extracted from tail snips of 9 – 14 day old animals. For standard DNA extraction, the tail snip was incubated overnight in DirectPCR™ Lysis Regent – Tail (Viagen Biotech, Los Angeles, USA) with 0.4 mg/ml proteinase K at 60°C in a shaking incubator. The proteinase K was deactivated by further incubation at 85°C for 45 minutes. The final composition of the PCR was 1x GoTaq® Green Master Mix (Promega, Sydney, Australia), 1 µM each respective primer and 1µL of DNA extract in 20µL volume. Bands were visualised after agarose gel electrophoresis by UV detection of ethidium bromide staining.

4.2.2 Generation of the 5xFAD crossed with IRAP KO

This project made use of a novel mouse stain that contained both the 5xFAD mutations as well as featuring the IRAP gene deletion. These IRAP KO mice were generated as previously described (Albiston et al. 2010). Briefly, fragments of the IRAP gene were isolated from mouse genomic DNA (C57Bl6) by PCR screening and a detailed exon-intro map of the IRAP gene obtained via

restriction digestions, southern blotting and sequencing. Using this, a targeting vector was constructed, containing Exon2 followed by the PGK-neo (a hybrid gene consisting of the phosphoglycerate kinase I promoter driving the neomycin phosphotransferase gene) marker, both flanked by *loxP* sites. This vector was then introduced into C57Bl6 embryonic stem cells via electroporation. Clones were obtained containing fragments characteristic for the intact and disrupted IRAP allele. Male chimeric offspring were bred with C57Bl6 female mice and germ line transmission of the mutant IRAP allele confirmed by Southern blotting. IRAPlox heterozygous male mice were mated with female Cre deleter mice (Schwenk et al. 1995). Female offspring with the IRAP(heterozygous)/Cre genotype from this mating were crossed with wild type male C57Bl6 male to remove Cre from the line. Heterozygous IRAP breeding pairs were then set up to produce offspring that featured; wild, type, heterozygous and homozygous knockout of the IRAP gene.

The breeding paradigm subsequently used in the generation of the mice for this study is described in (Figure 4-1). Five founder pairs (5xFAD males x IRAP KO females) were crossbred to produce the F1 generation. All of these animals were heterozygous for IRAP while half carried the 5xFAD mutations and half did not. Siblings from this generation were cross-bred to produce the F2 generation, leading to 6 possible genotype combinations as outlined in (Figure 4-1). Breeders were selected from this generation to produce two lines of mice that could be bred for IRAP homozygosity while still maintaining the production of both the 5xFAD transgenic and wild-type controls. At this stage, as the interest was only in the effect of the plaque coverage, this study made use of the 5xFAD with IRAP as its control animals to compare the effect of the IRAP KO on pathology in the 5xFAD mouse.

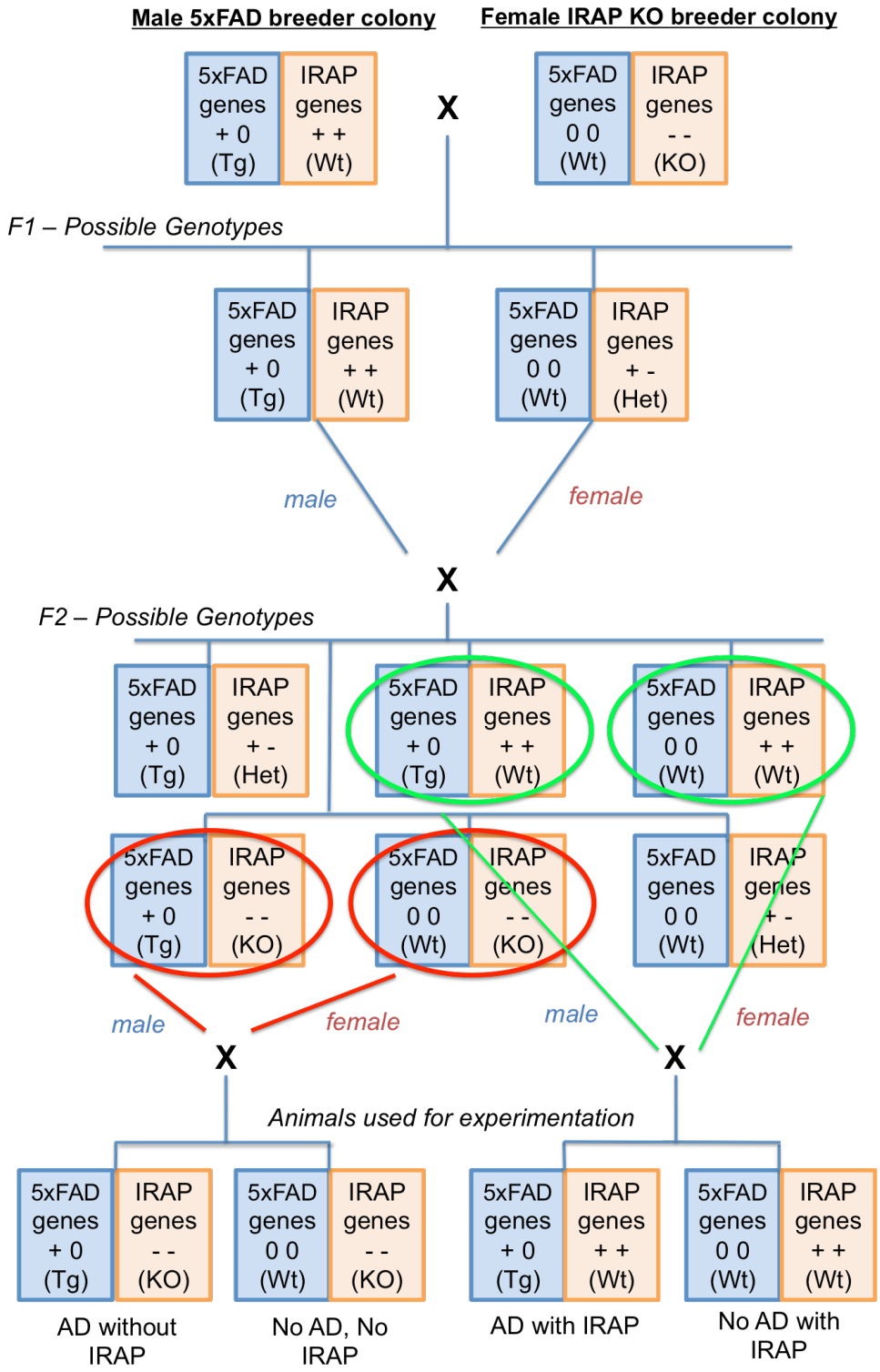


Figure 4-1 Schematic of breeding used to obtain mice featuring both 5xFAD mutations and the IRAP gene deletion

4.2.3 Quantification of plaque load

By 5 months of age the 5xFAD has established plaques within the subiculum and is beginning to show deposition in the retrosplenial cortex (Oakley et al. 2006) making these areas ideal to examine the potential disease modifying effects of IRAP deletion. Plaque coverage was assessed here as this is the predominant pathology these mice display and has been used a marker for therapeutic efficacy previously (Oddo et al. 2006). Mice were anaesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg) before being transcardially perfused via insertion of a needle into the left ventricle with ice-cold phosphate buffered saline (PBS; pH 7.3) and then ice-cold 4% (w/v) paraformaldehyde, 4% sucrose (w/v) in PBS. Animals were decapitated and the brains rapidly harvested before being placed in the same fixative for 24-hours, cryoprotected by immersion in 30% (w/v) sucrose overnight at 4°C and then snap frozen in isopentane on dry ice and stored at -80°C

Twelve-micron coronal sections were cut using a cryostat and thaw mounted onto gelatin-chrom-alum coated glass microscope slides. Sections were then placed in 30% Thioflavin S (Sigma-Aldrich) for 10 minutes before a 30 second immersion in 70% ethanol and three subsequent washes in PBS following which they were coverslipped with Mowiol mounting media (Sigma Aldrich). Bilateral images of the subiculum and retrosplenial cortex were taken on a Zeiss LSM700 inverted confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with luminosity and gain maintained at the same level across all sections. Analysis was performed as standard in the lab with the experimenter blinded to the genotype of the mice. ImageJ was used to threshold images to remove background staining before converting the images to grey scale and analysing the total area of coverage using the analyse particle function.

4.3 Results

4.3.1 Effect of IRAP gene deletion on plaque coverage in the 5xFAD mouse

In both the retrosplenial cortex and subiculum there was no difference between the genotypes in either the area of coverage (Figure 4-2 a,c) or the number of plaques (Figure 4-2 b,d). The level of pathology is what would be expected from the animals at this age (Oakley et al. 2006) and indicates that IRAP deletion does not affect the onset of pathology. It is conceivable that over the progression of the disease IRAP deletion could result in lowered pathology in the later stages, though there is no substantial evidence to support this notion, thus it was not investigated here.

Though these results do suggest that treatment with IRAP-Is would have little effect on plaque pathology in the 5xFAD. It is unlikely that this would be due to a developmental IRAP deficiency as plaque load was not exacerbated, there was simply no effect noted in the KO animals. In either case, this does not rule out use of the inhibitors as a potential treatment in human AD. While the classical understanding of AD paints amyloid as the primary causative agent of the disease (Selkoe 1991), and plaque coverage does form the primary endpoint for many studies (Dinkins et al. 2014, Kook et al. 2014), it should also be considered that many potential therapeutics (particularly those targeting aberrant pathological Aβ peptide production) fail in clinical trials (Mullane et al. 2013, Cummings et al. 2014) This indicates that perhaps the amyloid based mouse models are not accurately reflecting the human condition. As such it is important to investigate whether the mouse models replicate other features of human AD, particularly those that are relevant to IRAP-I mechanisms, namely glucose metabolism.

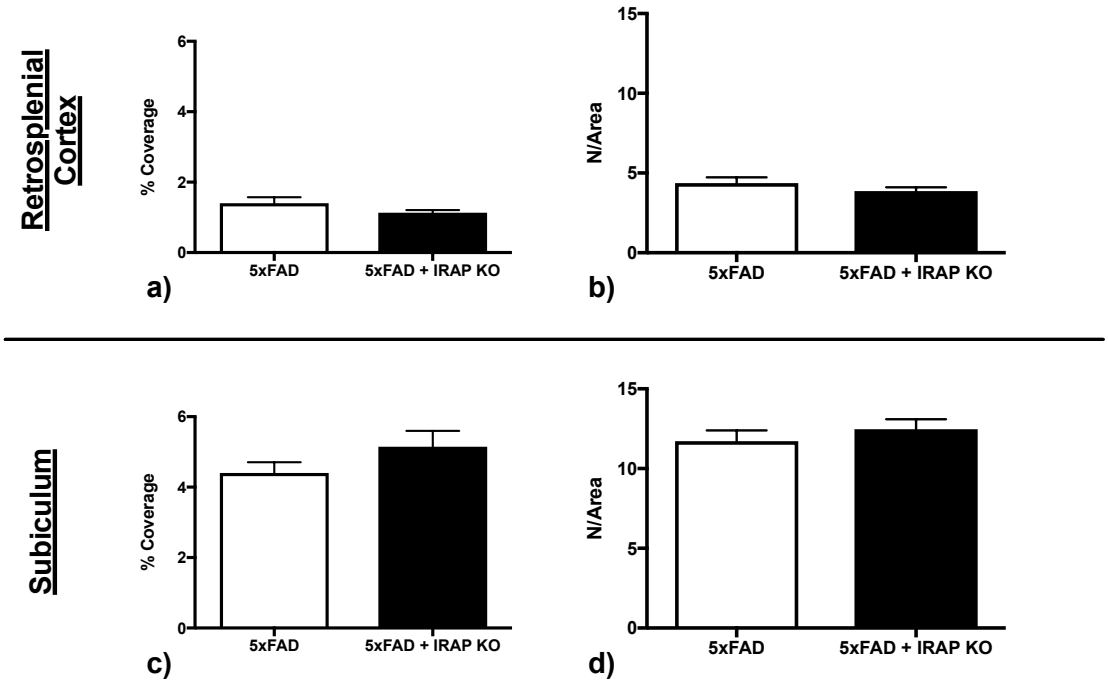


Figure 4-2 Plaque deposition in 5 month old 5xFAD mice crossed with IRAP knockout mice

There was no significant difference between in plaque coverage between the genotypes in either the a) retrosplenial cortex (with a plaque coverage of ~ 1.7%) (p = 0.1885) or c) subiculum (with a plaque coverage of ~4.2%) (p = 0.2071). In a similar fashion there was also no difference in the number of plaques in each area (b,d) (p = 0.2766 and 0.4325 respectively)

Data represented as mean ± SEM, n = 5/group, P value determined by two-tailed unpaired t-test

Part 2

4.4 Phenotype of Alzheimer's mice

Advances in neuroimaging using flurodeoxyglucose (FDG) positron emission tomography (PET) have demonstrated that persons with AD present with a specific pattern of progressive reductions in cerebral glucose uptake (CGU) (Mielke et al. 1996, Reiman et al. 1996, Herholz et al. 2002, Mosconi et al. 2005) that is now used as one of the diagnostic criteria for the disease (Dubois et al. 2014). FDG-PET has proven clinical diagnostic capability with the technique able to predict those who are most at risk of progressing from mild cognitive impairment to AD (Mosconi et al. 2004, Mosconi et al. 2008) and also able to differentiate between multiple dementia types (Mosconi et al. 2008).

However, while the pattern of CGU reductions in humans is well established, the evidence in the transgenic mouse models of the disease, particularly those that are solely amyloid based, is far less clear. While there are some similarities between the mouse and human conditions, such as a possible reduction in CGU in the posterior cingulate cortex (Valla et al. 2006), other studies have reported no change in key regions (Sadowski et al. 2004) or indeed even *increased* CGU (Poisnel et al. 2012, Rojas et al. 2013). As detailed earlier in this work, there are several possible explanations for these disparate results, with variations between studies including; animal sex, choice of normalization region, method of quantification and use of anaesthesia, all factors known to affect CGU. As identified in the previous chapter, increased glucose uptake is the likely mechanism by which the IRAP-Is mediate an increase in spine density and subsequent enhancement of cognition, thus whether the transgenic mice replicate the hypometabolism seen in the condition is critical to determine.

This lack of consensus regarding the exact phenotype of CGU in the AD mouse is critical to clarify, both for investigation of the IRAP-Is given their demonstrated interaction with glucose uptake, but also for the field at large regarding the translational applicability of the models. To date, there are no therapeutics available to treat AD progression despite repeated success in transgenic mice and this may due to the fact that plaque load does not correlate

with clinical presentation of memory deficits (Iqbal et al. 2014). Thus while novel therapeutics may be very successful in clearing plaque from the amyloid models, they may not treat the other potential underlying pathologies, such as reduced CGU, and this could explain their lack of efficacy in humans.

Another striking feature of human Alzheimer's is the neuronal death that takes place as the disease progresses, most notably affecting the hippocampus (Jack et al. 1999, De Santi et al. 2001), however this is not a consistently replicated feature in the AD mouse models (Wirths et al. 2010). Initial characterization of the 5xFAD model demonstrated neuronal loss in the subiculum and layer V of the cortex when assessed qualitatively (Oakley et al. 2006, Ohno et al. 2007), but not in the hippocampal CA regions. Later quantitative work confirmed these findings in layer V and the subiculum (Eimer et al. 2013), and also established that the hippocampal fields appeared to be spared (Jawhar et al. 2012). As well as overall neuronal loss in the human condition, there are also large reductions in dendritic spine density, particularly in the hippocampus (Ferrer et al. 1990, Ji et al. 2003). Reductions have been observed in the hippocampus of AD models such as the Tg2576 and PDAPP (Perez-Cruz et al. 2011), however in the 5xFAD animals it has currently only been observed in layer V of the cortex (Buskila et al. 2013, Crowe et al. 2014).

Inflammatory mechanisms are also closely associated with Alzheimer's disease, with the upregulation of several inflammatory mediators supposedly triggered by the cytotoxicity of amyloid-β peptides, particularly the most toxic species Aβ 1 – 42 (Salminen et al. 2009), which is also upregulated in the 5xFAD (Oakley et al. 2006). Given the possible interaction between the IRAP-Is and inflammatory modulation, particularly the expression of IRAP within astrocytes *(Fernando, Yeatman,* unpublished data*)*, this represents another possible mechanism by which the inhibitors may prove to be beneficial in Alzheimer's disease.

Thus, in order to phenotype the 5xFAD mouse with particular regards to factors that play a potential role in IRAP action, this study sought to determine if the 5xFAD mouse;

1) Shows the characteristic pattern of cerebral glucose uptake deficits, namely reductions in the posterior cingulate as well as hippocampus

To examine the effects pre-, mid- and late-disease progression, mice from 2 – 18 months of age were injected with radiolabelled glucose and brain glucose uptake levels quantified via autoradiography. As noted in Chapter 1 there are several methodological factors that may impede current research using the AD mouse models. This work attempted to eliminate these by using; autoradiography over PET, all male mice and delivering the radiolabelled glucose to conscious free moving animals, eliminating the confounding factors of anaesthesia and decreased body temperature.

2) Has a reduction in hippocampal area

Sections from the same animals underwent Nissl staining to visualise the hippocampus and manual measurement allowed quantification of hippocampal area at three levels; rostral, medial and caudal, in order to determine if hippocampal atrophy had occurred with disease progression.

3) Shows increased inflammatory cell activation

Staining for GFAP, a key protein highly expressed in astrocytes, and Cd11b, a key protein highly expressed in microglia, allows visualisation of the activation of each cell type respectively and can be used as a marker for inflammation and allow for a correlation with plaque coverage across age.

Despite the lack of effect noted on plaque pathology in the IRAP KO mice, there was still interest as to potential cognitive therapeutic benefit of the inhibitors. Global deletion of the IRAP gene from birth (Albiston et al. 2010) as well as post-natal forebrain specific knockouts (Yeatman et al. 2016) result in cognitive deficits, meaning that they are likely not suitable for assessing the cognitive potential of IRAP modulation in the 5xFAD mouse. Here I also decided to measure dendritic spine density following inhibitor treatment. There are currently few publications examining this phenotype in the mice, which would contribute to that literature. It would also build on the work from the previous chapters, not only allowing determination of the efficacy of the IRAP-Is in

rescuing a possible deficit in the AD mouse model but also providing the first *in vivo* evidence of the IRAP-I treatment enhancing dendritic spine density. Therefor the final aim of the study was to;

4) Examine the efficacy of peripheral IRAP-I administration at enhancing cognition and dendritic spine density in the 5xFAD mouse model

Following one month of inhibitor treatment delivered via minipump, memory of 5xFAD mice was assessed on three separate tasks. In a separate cohort of animals the Golgi-Cox stain was used to assess the density of dendritic spines in the CA1 region of the hippocampus and determine whether transgenic animals displayed reductions and if this could be rescued by IRAP-I treatment. As work from Chapter 2 suggested that IRAP-Is act to enhance normal memory, this protocol also allowed for examination as to the efficacy of peripheral IRAP-I delivery on dendritic spine density in wild-type mice.

4.5 Methods

4.5.1 Quantification of cerebral glucose uptake and brain slice collection

Mice were given intraperitoneal injections of 18μCi/100g body weight 2-[1-^{14}C]-Deoxy-D-glucose (PerkinElmer) in 0.9% saline and returned to their home cage in the dark for 45-minutes, a common dose and uptake period in the literature (Reiman et al. 2000, Valla et al. 2008, Nicholson et al. 2010). Following this they were deeply anaesthetised via isoflurane overdose after which a cervical dislocation was performed, they were decapitated, the brain rapidly removed and frozen in isopentane on dry ice before storage at -80°C. Sections were cut at 40μm on a cryostat and thaw-mounted onto gel-alum coated glass microscope slides. This yielded a minimum of 12 sections per animal that were used for analysis. Along with ^{14}C radioactivity standards (American Radiolabelled Chemicals) the sections were exposed for 2 -3 days to Kodak Biomax MR film (Sigma Aldrich) in light-tight cassettes. Films were developed by hand using GBX developer/fixer (Sigma Aldrich) and images collected in a CanonScan LIDE 90 flatbed scanner.

The experimenter was blinded to the genotype of the animals and the selected regions of interest were delineated manual before the mean grey scale value calculated using ImageJ (National Institutes of Health). Grey scale values were converted to levels of radioactivity on a log scale using the co-exposed standards.

4.5.2 Hippocampal cross-sectional area calculation

Following exposure to autoradiography films, the sections were Nissl stained by serial immersion of the slide mounted sections through progressively more dilute ethanol solutions (95 to 70 to 50%), followed by a wash in distilled water before 10 minutes in the cresyl violet stain. The sections were then washed in distilled water and dehydrated by serial immersion in progressively stronger ethanol solutions (50 to 70 to 95 to 100x) before finally being submerged in Xylene. Sections were mounted in DepeX mounting medium (Sigma-Aldrich) and the hippocampus imaged on a Zeiss LSM700 inverted confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The cross-sectional area of the hippocampus was quantified at three levels; -1.22, -2.54

and -3.52mm from bregma to obtain rostral, medial and caudal sections. The outline of the hippocampus from each hemisphere was delineated manually on ImageJ and area determined by the average of 2 sections per hippocampal level from a minimum of 3 animals per group.

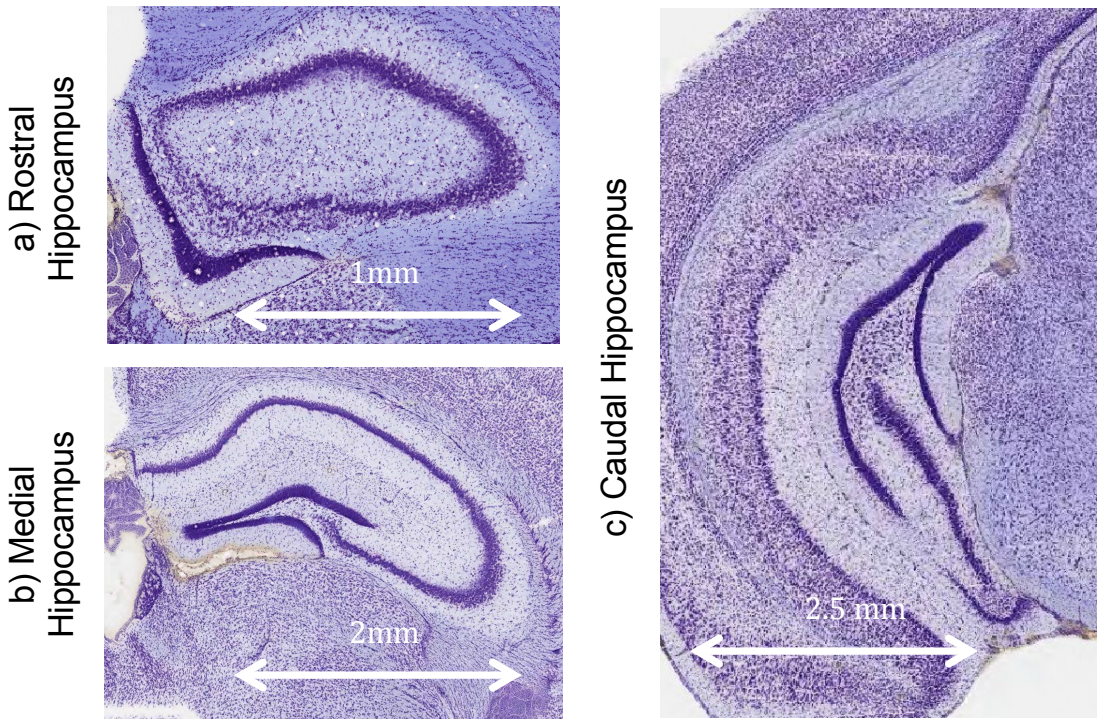


Figure 4-3 Nissl stained sections imaged for the calculation of hippocampal area in the 5xFAD mouse (5x magnification)

Images are representative of all animals that were imaged across both genotypes and all ages.

4.5.3 Quantification of plaque and astrocyte coverage

Sections were post-fixed for 10 minutes at room temperature with 4% (w/v) PFA in PBS and then washed twice with PBS before immunohistochemistry was performed. Sections were incubated with 10% (v/v) normal donkey serum in PBS with 0.3% (v/v) Triton-X-100 for one hour at room temperature to block non-specific binding of the antisera and permeabilize the cell membranes. Sections were then incubated overnight at 4°C with the primary antibody rat anti-GFAP to stain for astrocytes (1:500, Life Technologies) in PBS with 3% (v/v) normal donkey serum and 0.3% (v/v) Triton-X 100. Sections were then washed three times with PBS containing 0.1% (v/v) Tween-20 and the fluorescent-conjugated secondary antibody AlexaFluor 568 anti-rat (1:500, Introvogen) applied in PBS for two hours at room temperature. Following three washes in PBS with Tween sections were then placed in 30% Thioflavin S for 10 minutes before a 30 second immersion in 70% ethanol and 3 subsequent washes in PBS following which they were coverslipped with Mowiol mounting media.

The experimenter was blinded to the genotype of the animals and three images per mouse were taken of both hemispheres of the subiculum and retrosplenial cortex (-2.54mm to -2.7mm from Bregma) at 20x optical magnification on a Zeiss LSM700 inverted confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and image analysis performed in Image J as in 4.2.3.

4.5.4 HFI-419 preparation and Minipump implantation

As mentioned in the previous chapter, while HFI-419 is not detected in the brain 60 minutes following acute administration, it is rapidly detected in the minutes following administration. This means that continuous infusion via peripheral minipump is a viable option for chronic treatment. HFI-419 was dissolved in 100% DMSO and then diluted to a final concentration of 5mM in 30% w/v 2-hydroxypropyl-β-cyclodextrin (giving a final DMSO concentration of 30%) and allowed to equilibrate for one hour at 38°C. Vehicle control solution consisted of an equivalent volume of DMSO in the same solvent. This concentration was chosen in line with previous work from the lab that

demonstrated the efficacy of HFI-419 treatment in the reduction of plaque coverage in the AD mice (*Yeatman,* unpublished).

6-month-old wild type and 5xFAD male mice were randomly assigned to either vehicle control or drug treatment. Vehicle and HFI-419 solutions were loaded into osmotic minipumps (1004 model, 100µL volume, 0.11µl/hr delivery rate - Alzet, Cupertino, USA, Cat# 0009922) as per manufacturer's directions. Based on the average mouse weight of 30g this resulted in an hourly dose of 19.29 nmol/kg/hr. After loading, the pumps were primed for 24 hours in 0.9% w/v sterile saline at 37°C to initiate drug release. Mice were anaesthetised in 5% v/v isoflurane in oxygen and then maintained at 2% v/v. The back between the scapulae were shaved and swabbed with 70% ethanol. A small incision was made and pocket beneath the skin was formed with a pair of foreceps before the pumps were inserted and pushed towards the animal's rump and incisions closed with 7.5mm Michel clips. A subcutaneous injection of meloxicam was also administered as a post surgery analgesic. Bodyweight, appearance and other health characteristics were checked at 24 and 48 hours post surgery, and thereafter twice weekly.

5xFAD mice that were involved in the behavioural testing underwent minipump implantation by Peta Burns; I performed those that were used for quantification of dendritic spines.

4.5.5 Novel Object Recognition (NOR) task

The NOR task took place in dim diffuse lighting in 33 x 30 x 25 cm pink plastic boxes. Prior to the acquisition trial, mice were habituated to the testing box in three 10-minute sessions, each spaced 10 minutes apart before being returned to their home cage for 35 minutes. During the familiarisation trial, mice were placed in the box facing away from two identical objects that had been secured to the floor in adjacent corners (with enough room to allow complete movement around them) of the box. Mice were allowed 5 minutes to explore the objects and exploration time recorded, with exploration defined as the nose being less than 2 cm from the object when facing the object and actively engaging with it (sniffing, paw touching etc.). Climbing on the object was not considered as exploration. The boxes and objects were sprayed and wiped with

80% ethanol in between each animal to ensure the removal of all scent markings.

Here an inter-trial interval (ITI) of 30 minutes was chosen as previous evidence suggests this is sufficient to observe a deficit in memory in the 5xFAD mouse (Joyashiki et al. 2011, Tohda et al. 2011). After the ITI, one of the familiar objects was replaced with a novel object made from the same material but of a different shape replaced one of the objects. During this test trial, the mice were given 5 minutes to explore the box. The recognition index was determined as the time spent exploring the novel object minus the time spent exploring the familiar object, divided by the time spent exploring both objects. All video analysis was performed using TopScan™ software sourced from CleverSys. A subset of videos was also manually scored to confirm accuracy.

4.5.6 Y-maze tasks

The Y-maze consisted of grey Perspex (each arm 30 long x 9.5 wide x 15 high cm) and visual cues places around the room and periphery of the maze to act as reference points. As habituation to the testing arena is not possible in these tasks a small amount of sawdust from the animals home cage was scattered on the floor of the maze.

For the spontaneous alternation task the mouse is placed in the center of the maze facing one of the arms and allowed free exploration of the maze. An alternation is defined as entering all three arms within the space of three entries and the alternation score obtained by dividing this by the total number of possible alternations.

The novel arm preference takes place over two trials. During trial one, an arm is blocked off and the mouse is placed at the base of one of the remaining arms facing away from the centre. It is allowed to freely explore these two arms (termed home and familiar) for 10 minutes. The mouse is then removed for an ITI of 30 minutes. After this time the mouse is placed back in the maze with all three arms open for exploration. Novel arm preference is determined as time in the novel arm as a percent of total time in the novel and familiar arms.

4.5.7 Quantification of dendritic spine density

Brains were stained as standard using a RapidGolgikit (FD Neurotechnologies). As the recipes this staining procedure are proprietary I am unable to detail the precise contents of the solutions however golgi staining is common within the literature, particularly to visualise dendritic spines (Toy et al. 2014, Winston et al. 2016) and relies on the premise of impregnating neurons with a metal nitrate, typically silver, to allowing imaging of the neurons under a light microscope (Pannese 1999). Following one-month drug treatment, mice were deeply anaesthetized via isoflurane overdose and cervical dislocation performed before the brain was rapidly dissected out and placed in A+B solution for two weeks. Following this they were moved to solution C for 3 days before being snap frozen in isopentane on dry ice. 60µm sections were cut at -22°C and sections dried for 3 days at room temperature in the dark. Sections were washed in distilled water before being stained for 10 minutes in solutions D+E and progressively dehydrated before being cleared in xylene and mounted with DepeX. Second order dendrites in the CA1 region of the hippocampus (-1.58 to -2.18mm from Bregma) were imaged in vertical stacks (approximately 20µm thick) of 30 images under oil at 63x optical magnification on a Zeiss LSM700 inverted confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with a minimum of five basal and apical dendrites selected per animal.

4.5.8 Statistical Analysis

All analysis was performed in Graphpad Prism 6 and relevant statistic described under the figure as appropriate.

4.6 Results

4.6.1 Regional plaque coverage in the 5xFAD mouse across age

Four ages were chosen were chosen to assess plaque coverage; pre-symptomatic and pre-pathology (2 months), at the predicted beginning of pathology and behavioural disturbance (6 months), once pathology is supposedly well established (12 months) and towards the end of life (18 months). In line with previous examination of these mice (Oakley et al. 2006), there was an age-related increase in plaque coverage in both the subiculum ($p = 0.001$) where it reached a maximum of 15% and retrosplenial cortex ($P < 0.0001$) where it reached a maximum of 4% (Figure 4-6). As expected the subiculum showed plaque deposits even from 2 months of age (Figure 4-5) and also showed higher plaque coverage than the retrosplenial cortex at all ages, highlighting its particular vulnerability to plaque accumulation. Importantly this also allows for a comparison to be made between the onset of plaque pathology and potential deficits in glucose uptake.

In order to quantify CGU it was necessary to determine the most appropriate reference region to normalize glucose uptake against. Ideally, the reference region should have relatively low metabolic flux and also largely be spared from disease pathology. The cerebellum (Poisnel et al. 2012) and corpus callosum (Dodart et al. 1999) are commonly chosen within the literature and the fimbria was also chosen in this study due to its ease of identification. While all of the areas did show relatively little plaque accumulation in comparison to the subiculum and retrosplenial cortex (Figure 4-10) with maximum coverage of 1.5% at 18 months of age compared to the 15% in the subiculum, the cerebellum was an order of magnitude below the others and thus chosen as the reference region to normalize glucose uptake against.

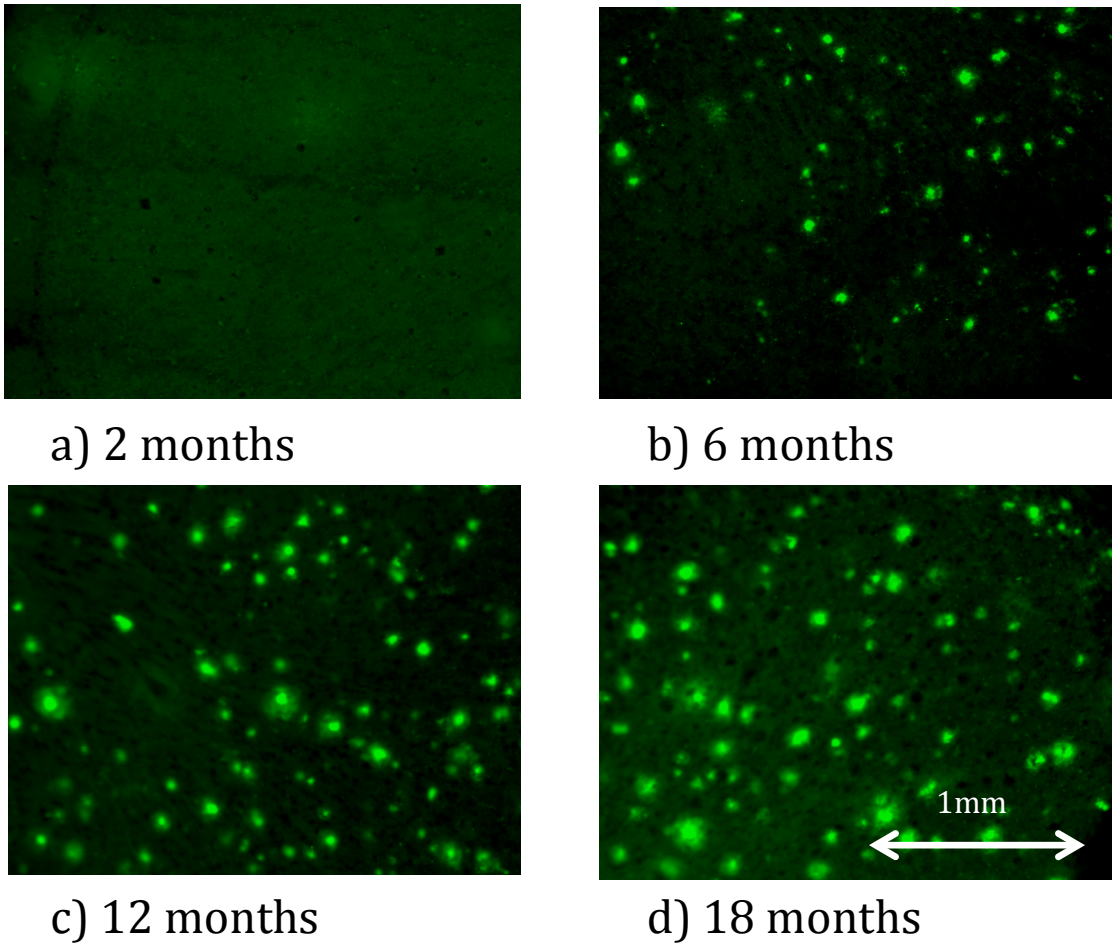


Figure 4-4 ThioflavinS staining indicating plaque accumulation in the retrosplenial cortex of the 5xFAD mice across age (5x Magnification)

Plaques are absent from the retrosplenial cortex at a) two months of age before appearing at b) six months of age. From here deposition increases throughout the rest of the animal's life with increased coverage noted at both c) 12 and d) 18 months of age.

Images representative of all animals at that age, n = 4 -5/group

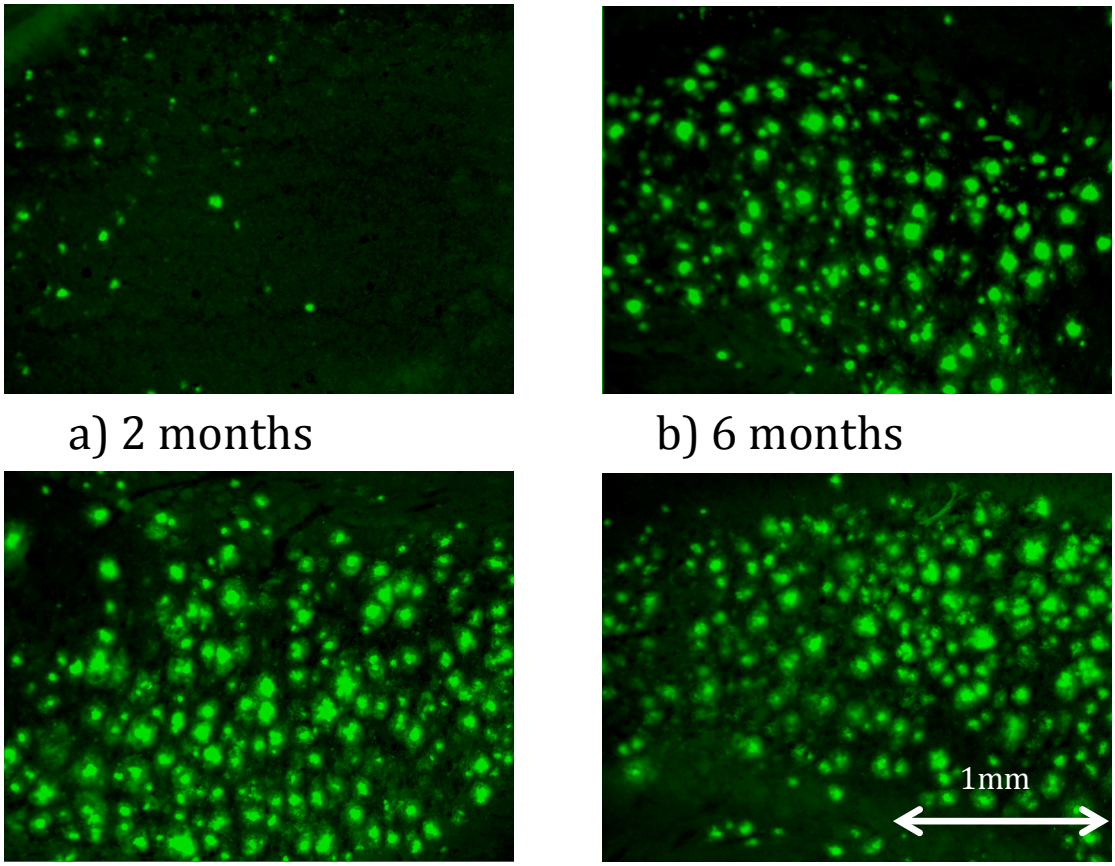


Figure 4-5 ThioflavinS staining indicating plaque accumulation in the subiculum of the 5xFAD mice across age (5x Magnification)

Highlighting the subiculum's particular vulnerability to pathology, plaques are noted even from a) two months of age. From here there is an increase with age through b) six , c) 12, and d) 18-months of age.

Images representative of all animals at that age, n = 4 -5/group

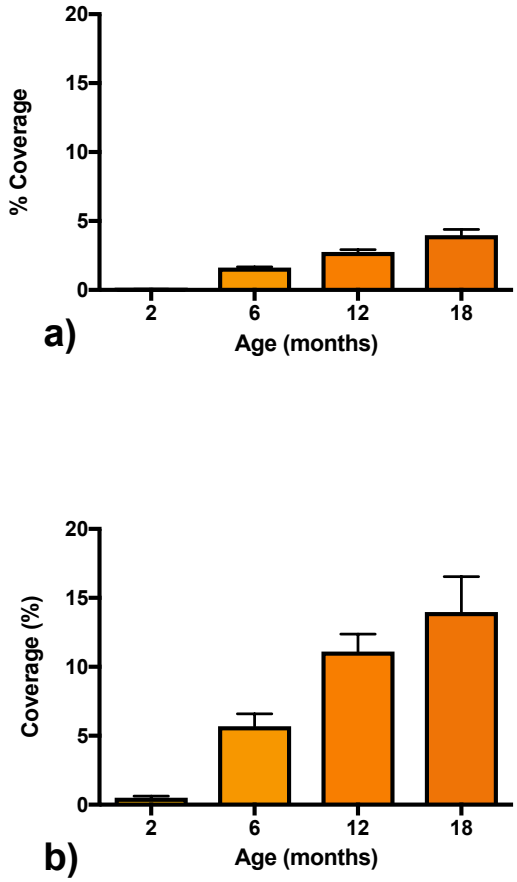


Figure 4-6 Plaque coverage in the retrosplenial cortex and subiculum of 5xFAD mice across age

Plaque coverage significantly increased with age in both the a) retrosplenial cortex ($p < 0.0001$, $F(3,7) = 46.46$) where it reached a maximum of 4% coverage, and the b) subiculum ($p = 0.001$, $F(3,8) = 15.87$) with a 15% maximum.

Data represented as mean ± SEM, n = 3 -4/group, P value determined by 1-way ANOVA testing for an effect of plaque coverage across age

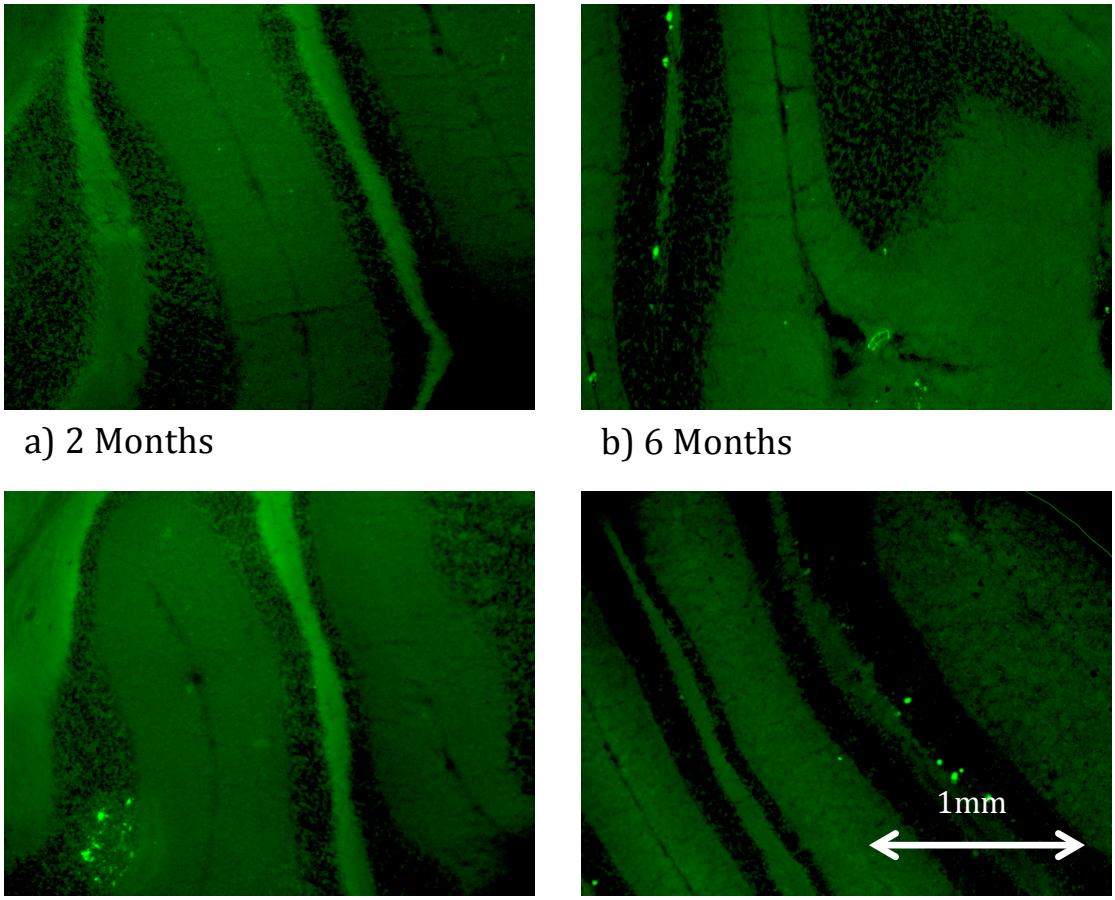


Figure 4-7 ThioflavinS staining indicating plaque accumulation in the cerebellum of the 5xFAD mouse across age (5x Magnification)

Plaques are absent from the cerebellum at a) two months of age before appearing at b) six months of age, albeit very sparsely. Deposition is stable throughout the rest of the animal's life with very few plaques present at both c) 12 and d) 18 months of age.

Images representative of all animals at that age, n = 4 -5/group

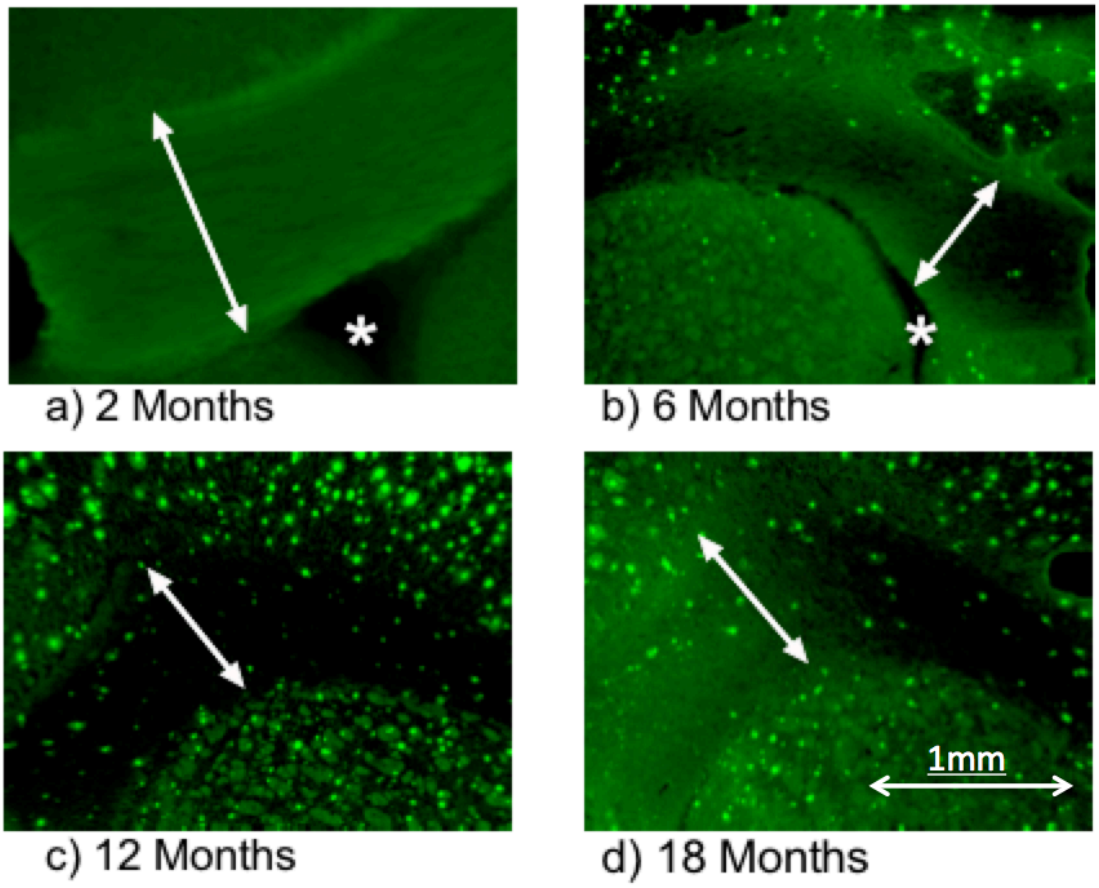
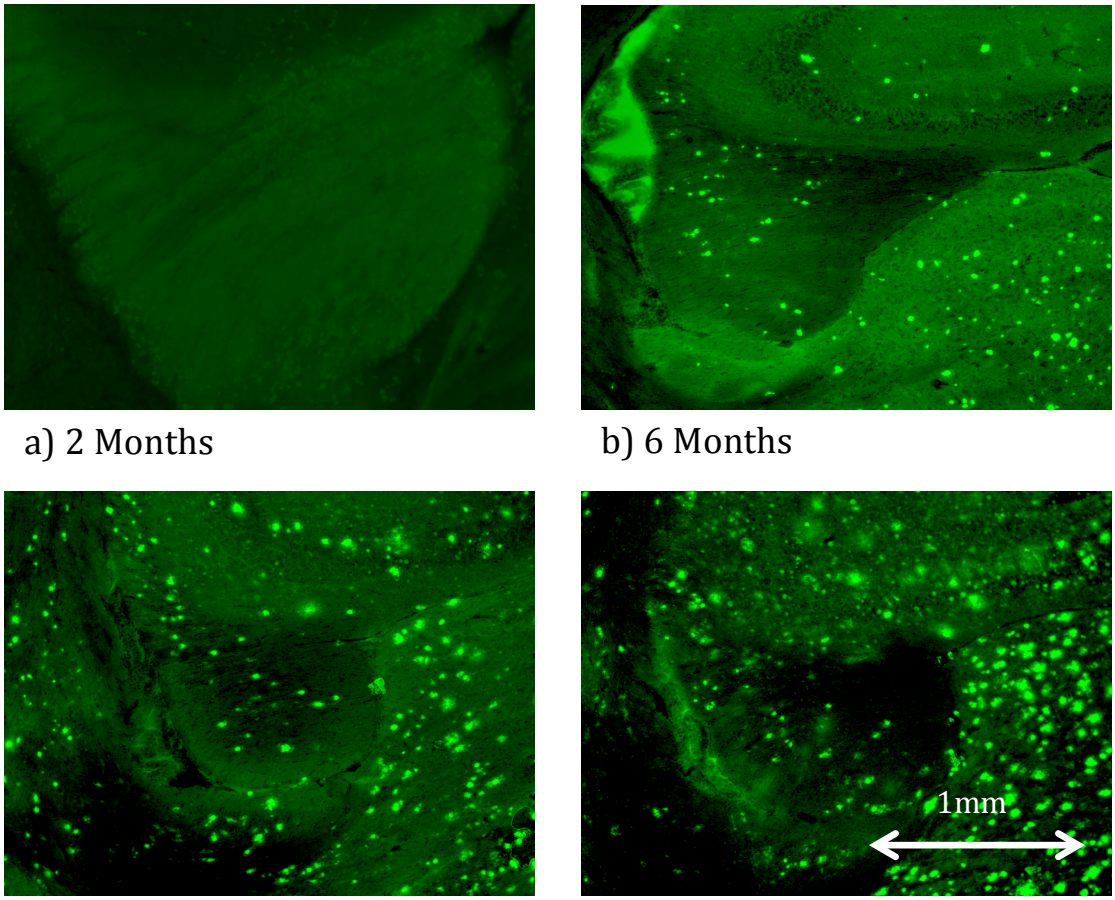


Figure 4-8 ThioflavinS staining indicating plaque accumulation in the corpus callosum of the 5xFAD mouse across age (5x Magnification)

There are no plaques present in the corpus callosum at a) two months of age. Deposition is apparent at b) six months, and increases at the c) 12 month time point and remains stable until 18 months of age. Images were taken throughout the length of the corpus, a) demonstrating rostral component, b) medial and c,d) further caudal regions. White arrows indicate the width of the corpus callosum itself and the asterisk (*) indicates the ventricle.

Images representative of all animals at that age, n = 4 -5/group



a) 2 Months

b) 6 Months

c) 12 Months

d) 18 Months

Figure 4-9 ThioflavinS staining indicating plaque accumulation in the fimbria of the 5xFAD mouse across age (5x Magnification)

As indicted by thioflavinS staining, there is no apparent plaque deposits in the fimbria at a) 2 months of age. By b) 6 months of age plaques have formed and persist at a stable level through c) 12 and d) 18 months.

Images representative of all animals at that age, n = 4 -5/group

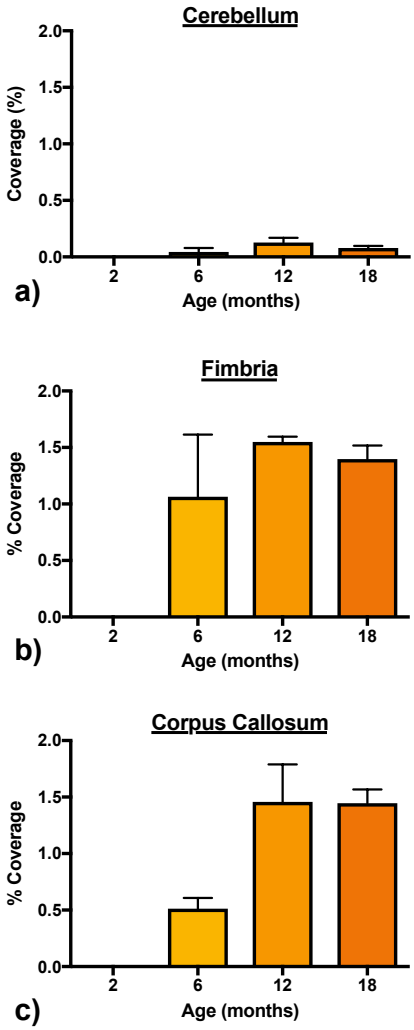


Figure 4-10 Quantification of plaque coverage in normalization regions across age in the 5xFAD mouse

All three areas are free of plaques at the 2-month time point. a) Cerebellar plaque deposition is ~10% that of the other regions. A small amount accumulates by 6 months of age and persists until 18 months, however this increase is not significant (p = 0.09, F (3,9) = 2.932). b) The fimbria has developed plaque deposition by 6 months of age, which remains stable until 18 months (p = 0.03, F = (3,7) c) The corpus callosum shows a small accumulation of plaque at 6 months age, rising to 1.5% by 12 months of age and remaining stable out to 18 months.

Data represented as mean ± SEM, n = 3 -4/group, P value determined by 1-way ANOVA testing for effect of plaque coverage across age

4.6.2 Regional glucose uptake in the 5xFAD mouse model across age

To determine if 5xFAD mice display the same hypometabolic phenotype as in the human condition, transgenic mice were compared to wild-type mice at 2, 6, 12 and 18 months of age. Multiple regions were assessed including the; hippocampus, cingulate and retrosplenial cortices, all regions known to susceptible in AD. Across all ages the only noted difference between the genotypes was in the thalamus at two months of age and the somatosensory cortex at 12 months of age (Figure 4-11 a and c respectively). The decision to analyse the results using multiple t-tests rather than an ANOVA was in line with previous autoradiogaphy studies (Reiman et al. 2000, Valla et al. 2006). Further, because only specific brain regions can be measured using autoradiography (in contrast to PET scanning which can measure total brain CGU), a significant genotype effect reported by a 2-Way ANOVA would erroneously suggest an overall genotype effect on brain metabolism. In contrast to the previous studies however, here I used Holm-Sidak correction for multiple comparisons and these are P-values reported in Figure 4-11. Based on the results of the plaque analysis discussed above, all values were normalised against cerebellar uptake.

	2 Month Old			6 Month Old		
Area	**p-Value**	**Wild-Type**	**5xFAD**	**p-Value**	**Wild-Type**	**5xFAD**
Hippocampus	0.53	0.94	0.85	1.00	0.91	1.01
Cingulate.C	0.11	1.5	1.4	1.00	1.48	1.46
Retrosplenial.C	0.11	1.6	1.5	1.00	1.42	1.51
Caudate	0.53	1.4	1.3	0.82	1.51	1.32
Granular.C	0.53	0.97	0.9	1.00	1.15	1.16
Entorhinal.C	0.77	0.87	0.85	1.00	1.06	1.10
Thalamus	**0.00003**	**1.7**	**1.4**	1.00	1.47	1.53
Piriform.C	0.51	1.2	1.1	0.73	1.52	1.31
Somatosensory.C	0.11	1.8	1.6	1.00	1.50	1.41
Auditory.C	0.53	1.5	1.4	1.00	1.46	1.55
Motor.C	0.11	1.5	1.3	1.00	1.36	1.35
Visual.C	0.17	1.5	1.4	1.00	1.42	1.43

	12 Month Old			18 Month Old		
Area	**p-Value**	**Wild-Type**	**5xFAD**	**p-Value**	**Wild-Type**	**5xFAD**
Hippocampus	0.61	1.03	1.08	0.87	0.99	1.01
Cingulate.C	0.17	1.51	1.63	0.59	1.72	1.64
Retrosplenial.C	0.07	1.50	1.66	0.64	1.77	1.70
Caudate	0.02	1.44	1.65	0.90	1.49	1.47
Granular.C	0.01	1.23	1.48	0.95	1.30	1.29
Entorhinal.C	0.07	1.17	1.33	0.56	1.15	1.24
Thalamus	0.05	1.54	1.72	0.62	1.69	1.62
Piriform.C	0.20	1.47	1.58	0.30	1.39	1.54
Somatosensory.C	**0.003**	**1.54**	**1.81**	0.83	1.60	1.64
Auditory.C	0.01	1.48	1.71	0.02	1.61	1.96
Motor.C	0.22	1.46	1.57	0.95	1.55	1.56
Visual.C	0.96	1.57	1.57	0.23	1.48	1.66

Figure 4-11 Regional glucose uptake (normalized against the cerebellum) across age in the 5xFAD mouse

All values presented as nCi/g and as a ratio against the cerebellum. a-d) Across all brain regions and ages, the only differences noted between the wild-type and 5xFAD mice were the thalamus at 2 months of age were a reduction was noted and the somatosensory cortex at 12 months of age were an increase was noted. (.C indicates cortex, e.g. Cingulate.C = cingulate cortex)

n = 5 – 6 group, P < 0.05 taken as significant and determined by multiple t-test with Holm-Sidak correction for multiple comparisons, comparing each region individually between genotype

4.6.3 Inflammation in the 5xFAD mouse model

At the commencement of this study there was a potential link between the IRAP-Is and a modulation of inflammation and given the inflammation present in AD this suggested another possible avenue for IRAP-Is to play a therapeutic role. To quantify inflammation in the 5xFAD mouse, coverage of astrocytes was examined in both the subiculum and retrosplenial cortex. Both regions demonstrated minimal coverage at 2 months of age before rising rapidly at 6 months of age and plateauing at this level, ~6% in the retrosplenial cortex and ~12% in the subiculum, through to 18 months (Figure 4-13). The lack of correlation between plaque and astrocyte coverage highlights their differential progression across age with both the subiculum ($p = 0.12$) and retrosplenial cortex ($p = 0.087$) demonstrating insignificant slopes.

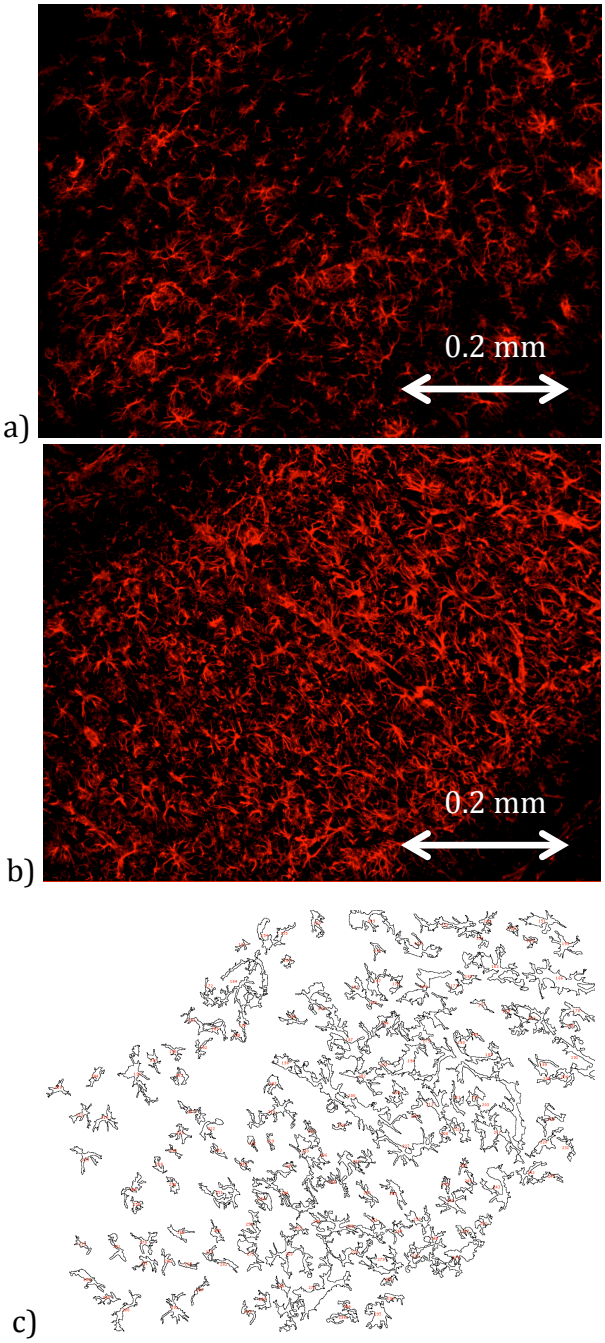


Figure 4-12 Representative GFAP staining for astrocytes in the hippocampus of 5xFAD mice (20x Magnification)

As indicated by the GFAP staining to visualise astrocytes within the hippocampus there is activation within both the a) retrosplenial cortex and the b) subiculum. c) represents the outline of those astrocytes that were quantified for coverage

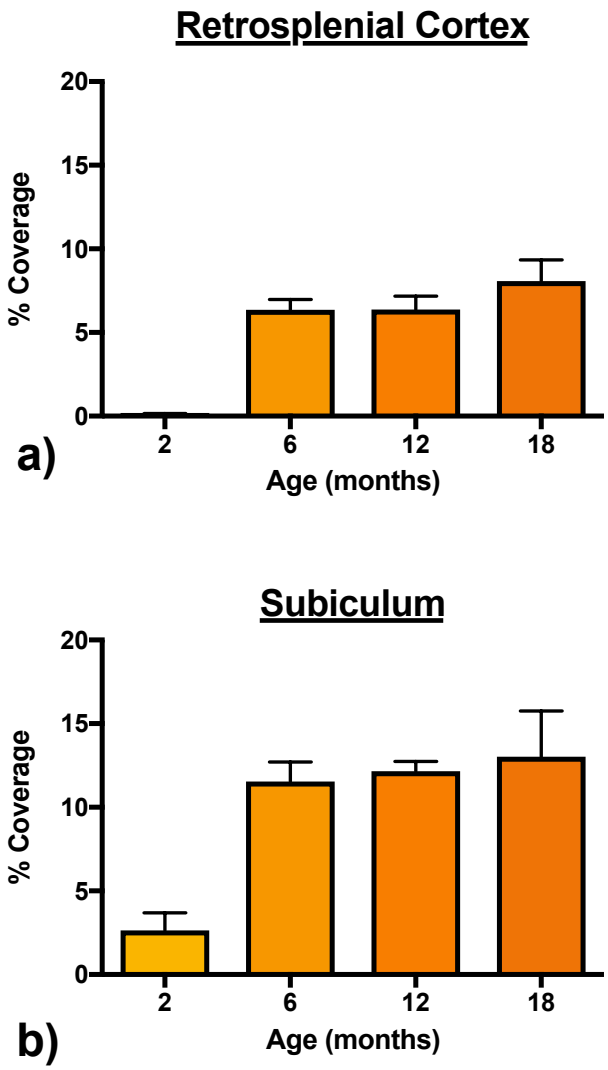


Figure 4-13 Astrocyte coverage in the retrosplenial cortex and subiculum of 5xFAD mice across age

Astrocyte coverage was initially low in both regions, at 2% in the subiculum before a large increase at 6 months of age and significantly increased with age in both the retrosplenial cortex (p =0.001, F (3,8) = 0.39), where it reached a maximum of 8% coverage, and the b) subiculum (p = 0.006, F (3,8) = 0.66) with an 13% maximum

Data represented as mean ± SEM, n = 3 -4/group, P value determined by 1-way ANOVA examining astrocyte coverage across age

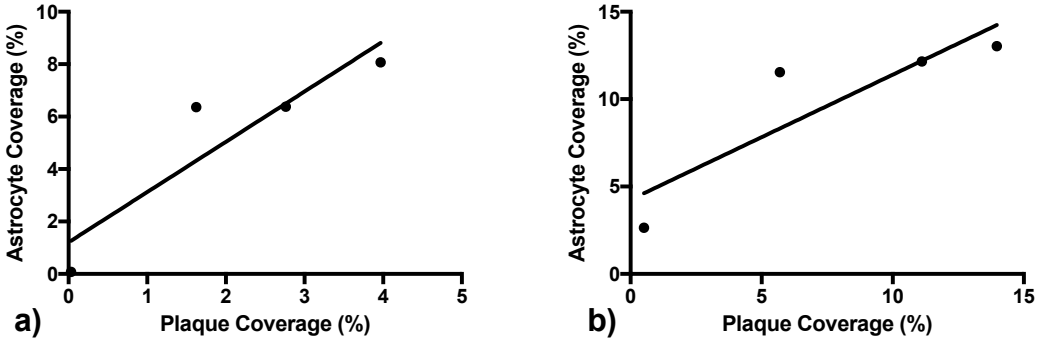


Figure 4-14 Correlation of plaque and astrocyte coverage in the retrosplenial cortex and subiculum of 5xFAD mice across age

While plaque coverage increases in a linear fashion with age, astrocyte coverage rapidly elevates and plateaus at 6 months of age. Correlation determined by linear regression demonstrated a lack of significance in both the a) retrosplenial cortex (p = 0.087) and b) subiculum (p = 0.12).

Data represented as mean of the respective measurement, n = 3 -4/group, P value determined by analysis of linear regression.

4.6.4 Hippocampal area of the 5xFAD mouse

Inhibition of IRAP activity has been shown to increase neuronal survival in pathological states such as ischemic stroke (Pham et al. 2012) and neuronal loss is a key feature of human AD (Šimić et al. 1997). As neuronal death has already been shown to be very minimal or absent in the 5xFAD mouse hippocampus (Jawhar et al. 2012, Saul et al. 2013), cross-sectional area to estimate overall atrophy was quantified here. Ideally overall volume would have been calculated, however due to the effect the golgi impregnation has on tissue stability when collecting sections on the cryostat I was unable to obtain a complete sampling of the hippocampus, thus three levels were chosen instead. As hippocampal atrophy forms one the earliest and most striking features of the human condition (Frankó et al. 2013) this would provide clues as to whether the size of the hippocampus is reduced due to other factors, such as extracellular matrix degradation for example and would warrant further investigation. As expected there was an increase of hippocampal volume with age, however similar to CGU, no differences were noted between either the wild-type or transgenic mice at any age (Figure 4-15).

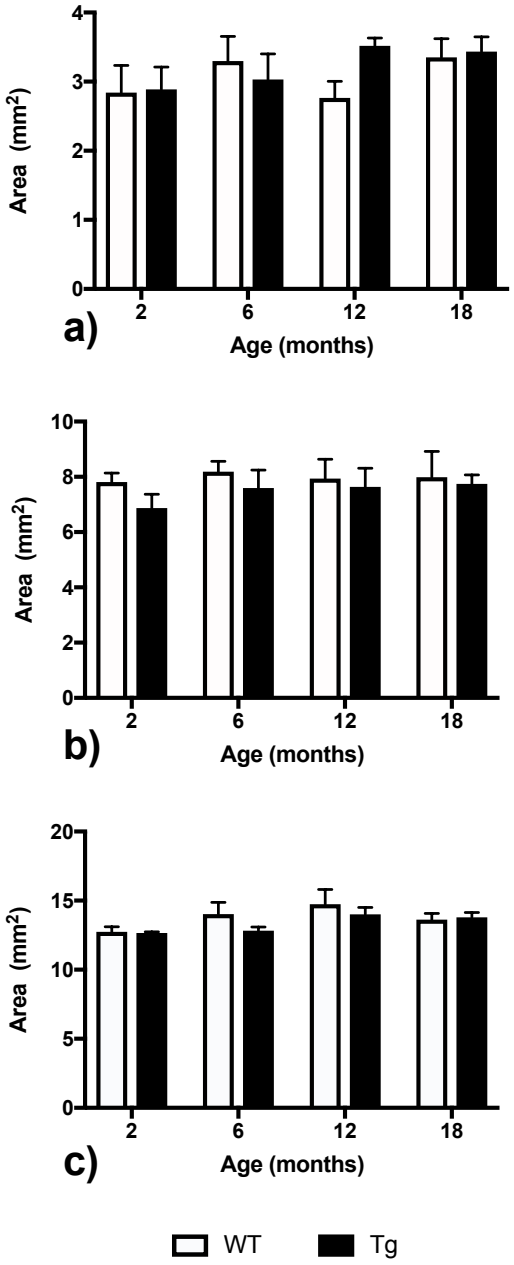


Figure 4-15 Comparison of hippocampal cross sectional area between control and 5xFAD mice across age

There was no difference between cross sectional area of the hippocampus across age or between genotype in the a) caudal (-3.52mm) b) mid- (-2.54mm) or c) rostral (1.22mm) hippocampus.

Data represented as mean ± SEM, n = 3 - 6/group, P value determined by 2-way ANOVA with age and hippocampal volume as factors

4.6.5 Performance of wild type and 5xFAD mice treated with HFI-419 on cognitive tasks

In order to examine the efficacy of IRAP-I treatment on rescuing the cognitive deficits caused by Alzheimer's disease, 5xFAD mice were treated with HFI-419 via subcutaneous minipump for one-month before being assessed in three common behavioural memory tasks. In this work, no significant effect of either genotype or treatment was noted in the novel object recognition task (object recognition memory), free roaming y-maze (spatial working memory) or spatial recognition y-maze (spatial recognition memory). Notably, in the first two tasks, control animals performed at chance levels (0 RI and ~50% alternation respectively) while in the spatial recognition y-maze the transgenic animals performed above chance, reaching scores of ~65%. Mice performed the same number of total alternations in the y-maze task indicating that locomotion was unlikely to be different between the groups. While a direct comparison could have been made between the transgenic HFI-treated and untreated mice, a One-way ANOVA with multiple comparisons was chosen to examine this data. This was because there was interest as to whether the transgenic animals display a deficit compared to the wild-type animals, as well as whether there is an improvement with HFI-419 treatment.

(*Surgery and behavioural testing of this cohort of mice performed by Peta Burns)*

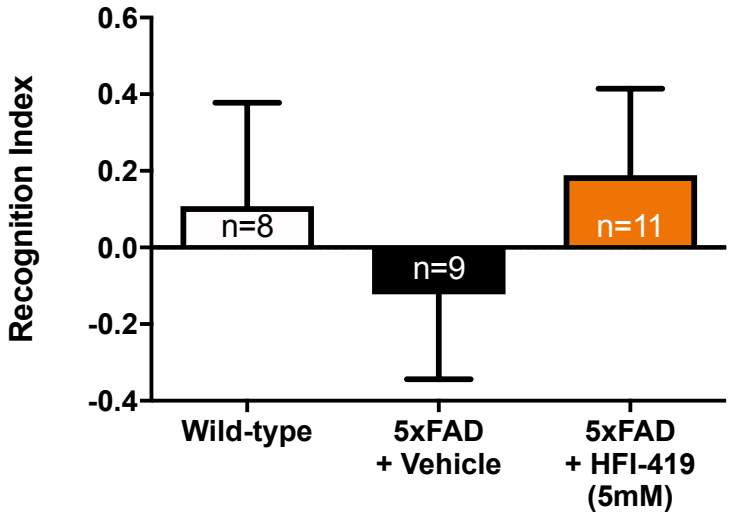


Figure 4-16 Recognition index of performance of wild-type and transgenic mice in the Novel Object Recognition task

Mice in each of the groups performed around chance levels. Slight tendency towards the novel object was noted in the wild-type and treated groups and a slight tendency towards the familiar object in vehicle treated group, however none of these results were conclusively different from zero nor was there a significant difference between the groups ($p = 0.6289$, $F(2, 25) = 0.01784$).

Data represented as mean ± SEM, P value determined by 1-way ANOVA with Tukey correction for multiple comparisons testing across groups for a difference between recognition indexes.

Work performed by Peta Burns

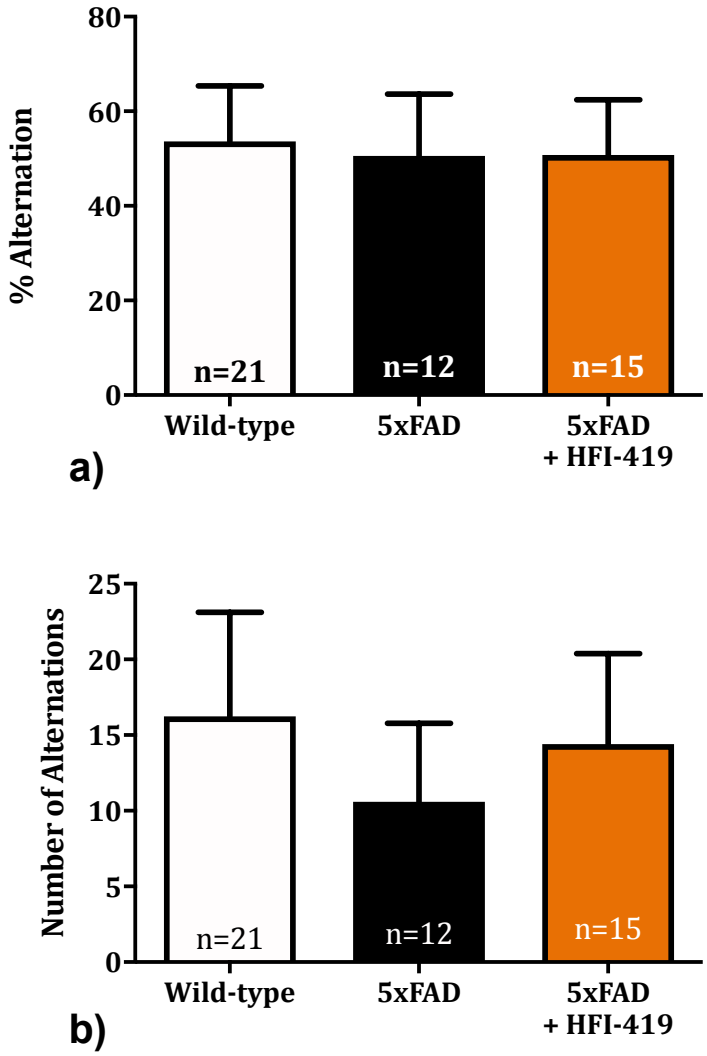


Figure 4-17 Alternation performance of wild-type and transgenic mice in the y maze task

a) *Each of the groups performed close to chance levels (~50%) and there was no significant difference in performance between any of the groups (p = 0.7018, F (2, 45) = 0.1661). b) There was also no significant difference in total number of alternations between the groups indicating no difference in locomotion (p = 0.0522, F (2, 45) = 0.4482).*

Data represented as mean ± SEM, P value determined by 1-way ANOVA with Tukey correction for multiple comparisons testing across groups for a difference between alternation scores/arm entries

Work performed by Peta Burns

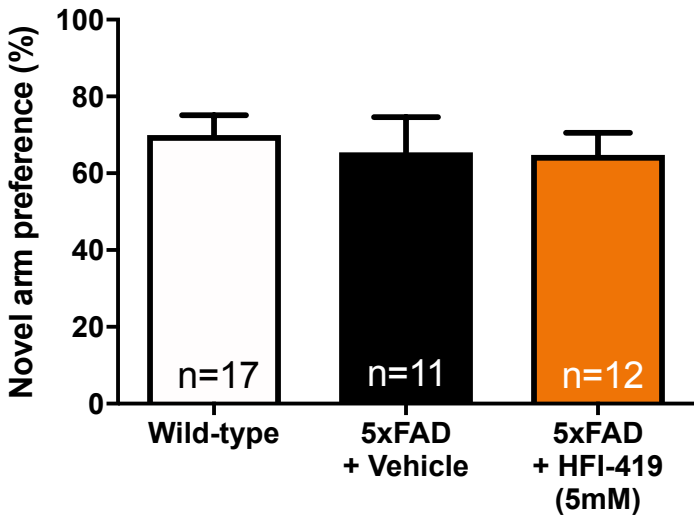


Figure 4-18 Novel arm preference of wild-type and transgenic mice in the Y-maze task

Each of the groups performed above chance (65 – 70%) indicating successful memory retrieval of the familiar arm, however there was no difference in exploration time between any of the groups (p = 0.8148, F (2, 37) = 0.7215).

Data represented as mean ± SEM, P value determined by 1-way ANOVA with Tukey correction for multiple comparisons testing across groups for a difference between novel arm preference

Work performed by Peta Burns

4.6.6 Spine density and the effect of HFI-419 treatment in the 5xFAD mouse

Despite the lack of effect on plaque coverage seen in the 5xFAD following IRAP deletion there was still interest as to the *in vivo* effect of the inhibitors on dendritic spine density. As discussed previously, dendritic spines are a cellular correlate of memory and show large reductions in AD, particularly within the hippocampus. Pilot data conducted previously in the lab within a different strain of AD mice, the APD9 model, had suggested that treatment with HFI-419 was able to enhance dendritic spine density, however the transgenic animals themselves did not display a reduction compared to wild type. This work was hindered due to low sample size, poor image quality and also not including a treatment group of the wild type animals.

The current work expanded on this pilot data by using the 5xFAD mice to further phenotype the model as well as including a wild-type treatment group. The CA1 region of the hippocampus was chosen as it is particularly vulnerable in human AD patients (Ardent 2001) and has previously been demonstrated to be affected in the transgenic mouse models in regards to dendritic spine density (Perez-Cruz et al. 2011). Five – six month old mice were used in this study as animals are beginning to show cognitive deficits (Oakley et al. 2006) and thus should also show reductions in dendritic spine density. Comparing first the genotypes, dendritic spine density was reduced in the CA1 region of transgenic mice compared to the wild-type animals (Figure 4-19), in line with previous evidence from the mouse models (Perez-Cruz et al. 2011). Regarding HFI-419 treatment, one-month dosing resulted in an increase in spine density in the CA1 region of both the transgenic and wild-type animals (Figure 4-19). Ideally plaque coverage would have also been quantified in these animals however the golgi-cox stain precluded the co-staining for plaques.

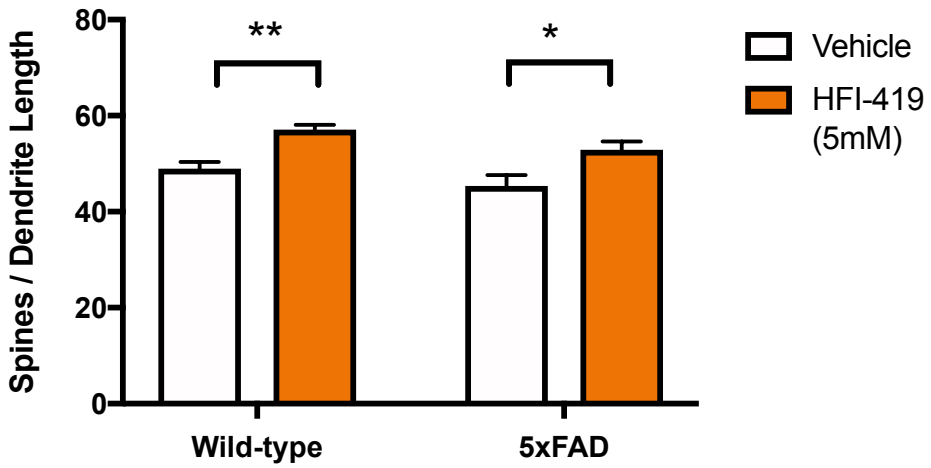


Figure 4-19 Spine density in region CA1 of the hippocampus in 5-month-old 5xFAD mice treated with HFI-419 (5mM dose) via subcutaneous minipump for one month

Comparing between the genotypes there was a reduction in spines in the 5xFAD animals compared to the wildtypes ($p = 0.032$, $F(1,16) = 5.494$). Spine growth was enhanced in response one-month treatment with the 5mM dose HFI-419 at the pooled level ($p = 0.0002$, $F(1,16) = 22.17$), in both the wild-type ($p = 0.0063$) and the 5xFAD ($p = 0.011$) animals. There was no interaction ($p = 0.84$, $F(1,16) = 0.04$)

*Data represented as mean ± SEM, n = 5/group, * P < 0.05, ** P < 0.01, P value determined by 2-way ANOVA with genotype and treatment as factors. Post-hoc multiple comparisons comparing between treatment groups with Sidak correction*

4.7 Discussion

Following the lack of effect noted on plaque coverage in the 5xFAD mice featuring the IRAP gene deletion the main focus of the study became to phenotype the 5xFAD mouse model with particular regard to factors that likely to interact with IRAP inhibitor action; namely glucose uptake, hippocampal atrophy, inflammation and dendritic spine density. Compared to wild-type animals CGU was largely unchanged in the 5xFAD mice with the only reductions noted being at 2 months of age in the thalamus and 12 months of in the somatosensory cortex, neither of which persisted beyond this age. Cross sectional area of the hippocampus was also unchanged at three levels indicating that overall atrophy was unlikely to have occurred. While an upregulation of inflammation was noted with age the 5xFAD mice, this increase occurred between 2 – 6 months of age and plateaued at this level, indicating it did not worsen with age nor did it correlate to plaque load. The mice did show a reduction in dendritic spine density within the CA1 region of the hippocampus, similar to the results noted in the human condition.

Following this, treatment of the 5xFAD with HFI-419 via subcutaneous minipump was able to enhance dendritic spine density in both the wildtype and 5xFAD mice, indicating both the capacity to rescue deficits induced by the disease process, but also, as predicted by the previous chapters, enhance growth in normal animals.

4.7.1 Cerebral glucose uptake in the 5xFAD mouse model across age

While the human condition of Alzheimer's is characterized by regional glucose deficits, commencing and most pronounced within the hippocampus, posterior parietal and retrosplenial cortices (Nestor et al. 2003, Mosconi et al. 2008), the literature is far more conflicted as to the phenotype in the AD mouse models. This is particularly exacerbated in the 5xFAD model due to current studies having only been performed with PET scanning, as detailed previously, the resolution of current PET scanners is above the size of spatially small, but very relevant areas, such as the hippocampus and this limits the conclusions that can be drawn from these studies.

Using autoradiography in this study, it was noted that there was no regional deficit in glucose metabolism across any of the examined regions, except the thalamus at two months of age and somatosensory cortex at 12 months, and this was apparent across all of the ages examined. While an increase in thalamic CGU has been noted (Luo et al. 2012), the majority of AD mouse studies demonstrate a reduction in thalamic CGU (Dodart et al. 1999, Niwa et al. 2002, Dubois et al. 2010), though even in these cases the exact timing is unclear, some showing a reduction from early ages while in others it does not appear until late life. In the somatosensory cortex a reduction has also been noted in some studies (Valla et al. 2002, Dubois et al. 2010), however studies have also reported no change (Valla et al. 2006) or indeed even hypermetabolic within the same model (Valla et al. 2008). Why these changes are only reported at a single time point in the current study is unclear, however this is not an uncommon phenomenon in the assessment of CGU in AD mice

Speaking to the human condition, initial characterisation of human AD suggested that the thalamus was only minimally affected in the early stages of the disease (based on the deposition of plaques) (Braak et al. 1991). More recent work has indicated that the thalamus may be vulnerable to atrophy even in the preclinical and MCI stages of the disease (Yi et al. 2015, Kälin et al. 2017). Whether CGU is affected is contested, with some studies reporting that it is spared (Li et al. 2008, Marcus et al. 2014) while others have shown reductions early on (Hunt et al. 2007, Castellano et al. 2015). There is relatively little discussion of the somatosensory cortex in the literature in regards to hypometabolism, however overall it appears to be largely spared until the very late stages of the disease (Chételat et al. 2008, Marcus et al. 2014).

4.7.2 Comparison with previous studies in the 5xFAD mouse

To my knowledge there have been three studies that have directly examined cerebral glucose uptake in the 5xFAD mouse model. In the first, 12-month-old mice showed an overall elevated CGU in the cortex relative to the cerebellum (Rojas et al. 2013) while another study directly contrasted this demonstrating that 13-month-old 5xFAD mice displayed diminished CGU compared to wild-type mice (Macdonald et al. 2014). Interestingly, this work by

Macdonald and colleagues appeared to be due to an unexpected age-related elevation in the wild-type CGU while the transgenic animals remained constant. Normalization of every brain region against each other lead to several areas appearing to have reduced uptake at both 5 and 13 months of age, while there was increased uptake at 2 months. These results should be interpreted with caution, as there is no explanation given as to relevance of ratios between what are in some cases structurally and functionally distinct brain regions. In the most recent study, initial hypometabolism was noted in the olfactory bulb at 3 months before progressing to the cortex and hippocampus by 6 months of age (Xiao et al. 2015). At present it is difficult to draw definite conclusions regarding the CGU phenotype of the 5xFAD mouse given how disparate the current studies are. The present study could be regarded as more accurate given that it makes use of autoradiography as well as addressing several other methodological issues (discussed below).

This study has aimed to combat factors that could have confounded previous studies (as outlined earlier in the thesis). This was achieved by using the more spatially sensitive autoradiography technique, avoiding the use of anaesthesia until the mice were killed, allowing free roaming activity during the uptake period and by only using male mice. Broadly these results add to the literature demonstrating that the Alzheimer's mouse models do not effectively replicate the human progression of cerebral hypometabolism. There does remain the possibility that amyloid can indeed cause CGU deficits and simply requires a longer time frame to induce this neuronal dysfunction, amyloid being present in the human brain for years to decades like in the human condition for example as opposed to the roughly 18 months the mouse models provide.

Overall however, this phenotype of CGU does not follow the progression noted in human condition and this is particularly important for the action of the IRAP-Is. It has been suggested that hypometabolism can exacerbate the deposition of amyloid plaques (Velliquette et al. 2005, Mosconi et al. 2008), therefore the lack of hypometabolism seen here could explain why no effect was noted in the IRAP KO mice. Importantly however, this does not preclude the potential use of the inhibitors from use in human AD. As mentioned, the *human*

condition is defined by an extensive and progressive reduction in CGU. Thus there still exists the possibility for the inhibitors to play a therapeutic role, however it is difficult to determine this with the mouse models that are currently available. Of note is that the interaction between tau and GLUT4 is unclear. One possibility is that a tau-mediated reduction in GLUT4 would reduce the efficacy of the IRAP-Is by reducing the transporters available for recruitment to the membrane. Contrary to this, IRAP-Is may be able to overcome to reduction mediated by tau and serve as an effective therapeutic in this case. In AD there is reduction in both GLUT1 and 3 (Liu et al. is associated with a reduction . In either case, investigation in tau bearing models is also prudent.

4.7.3 Choice of Reference Region

Another goal of the current study was to determine which area of the brain is the most suitable to serve as a reference point to normalize CGU against. Previously the corpus callosum (Dodart et al. 1999), cerebellum (Sadowski et al. 2004) and whole brain average (Reiman et al. 2000) have been used. Here the fimbria was also examined due to the ease of identification and as white matter tract, predicted to be largely free from amyloid pathology. This work suggests the cerebellum is the most suitable region to normalize against based on the relatively small accumulation of plaques compared to the corpus callosum and fimbria.

While whole brain average has been used previously (Reiman et al. 2000), it is unlikely to be suitable in an AD model. Firstly, whole brain averages are often used in cancer diagnosis because the cancer is a small and isolated pathology and the metabolism of other brain regions is not affected. In a disease such as AD however, pathology is diffuse and spreads throughout the brain while also affecting CGU broadly (Mosconi et al. 2009). Further, while a PET scan can capture the entire uptake of the brain, using autoradiography it would be necessary to examine every single region while also accounting for relative sizes. Though it is possible to capture many important regions, such as the hippocampus and thalamus for example, there are numerous other areas that could be affected. Regions are often also chosen specifically because they are predicted to be affected in the course of the disease. In the work by Reiman and

colleagues (2000) for example, the average activity of 70 regions is used to create a mean to normalize against, however this does not account for either of the two problems mentioned. As such, a whole brain average is not suitable for CGU normalization of autoradiography.

4.7.4 Lack of correlation between inflammation and plaque coverage

There is tentative evidence to suggest that IRAP is expressed in activated astrocytes (*Yeatman, unpublished)* and that IRAP-Is may be able to modulate inflammation and reduce cortical damage in rats undergoing ischemic stroke (*Teliandis, unpublished)*. As one of the mechanisms by which amyloid is suggested to have its detrimental effects on the brain is through the upregulation of several inflammatory mediators such as TNF-α and NF-κB (Wyss-Coray 2006) there was initially interest as to the phenotype of the AD mice in this regard.

In line with this, it has been suggested that treatment with anti-inflammatory agents can reduce AD pathology in the 5xFAD mouse, although there is conflicting evidence to this notion. Treatment with peroxisome proliferator activated receptor (PPAR) δ agonists has been reported to reduced amyloid pathology and reduce overall microglia activation and inflammatory mediator expression (Kalinin et al. 2009, Malm et al. 2015). PPAR-γ agonism also has positive effects on AD markers in mouse models of AD. This is particularly interesting in the context of this thesis, as ARBs such as telmisartan have been reported to have their positive effects through partial PPAR-γ agonism (Mogi et al. 2008, Tsukuda et al. 2009). In contrast to these results, treatment with ibuprofen reduced inflammation but had no effect on plaque pathology and actually exacerbated behavioural deficits (Hillmann et al. 2012).

While these results may suggest that is possible to reduce plaque burden in AD mice though the use of anti-inflammatory agents and it is indeed well accepted that there is an increase activation of astrocytes in 5xFAD mouse brain (Rojas et al. 2013, Kook et al. 2014) and that there may even be a close correlation of astrocyte activation with plaque density (Bhattacharya et al. 2014), these studies have only examined animals at a single time point. In the PS2APP transgenic mice there was an abrupt doubling of microglial expression

in the subiculum at 13 months of age that persisted at 17 months (Richards et al. 2003) highlighting that inflammation did not increase in step with amyloid burden.

To my knowledge there is only a single study that tracked the changes in inflammatory activation across time in the 5xFAD model. Here inflammatory mediator expression was low at two months of age before showing a very large increase at four months of age that was sustained at 6 months. This was true for both GFAP expression and the cytokine Il-1β (Py et al. 20014). As the PS2APP is generally regarded as a less aggressive model compared to the 5xFAD the later onset of inflammation is not surprising, importantly however is the disconnect between plaque pathology and inflammation. Both of these studies are similar to the work presented here that demonstrates that while plaque coverage followed a linear increase with age, astrocytes rapidly increase from 2 to 6 months of age and then remain at this level.

This is an important distinction as the interest here is not whether the mice show inflammation similar to the human condition, but whether the progression of these markers is the same. Overall, this rapid upregulation of astrocytes appears to run counter to the human condition where it is suggested the primary event is the deposition of plaques (Akiyama et al. 2000) that in turn drive the upregulation of the inflammatory mediators and that inflammation levels are correlated with pathology (Eikelenboom et al. 2006). While inflammatory cytokines were not directly measured here, this by no means makes astrocyte coverage a proxy marker. Coverage is known to increase in AD (Vijayan et al. 1991) and increased expression of GFAP is one of the few similarities that human AD and the mouse models share (Wyss-Coray 2006). Nonetheless, given that immune cell coverage precedes plaque deposition here it is unclear what is driving the upregulation. Human astrocytes become activated in response to aggregated amyloid (Pike et al. 1994), however it is possible that mouse astrocytes are responding to increased levels of soluble amyloid.

Where this leaves treatment with the IRAP-Is is unclear. While this early rise inflammation, preceding plaque coverage, does offer a window to begin

drug administration with the goal of reducing plaque coverage and cognitive decline, the utility of treating with the IRAP-Is is unclear. By the conclusion of this study further work from colleagues in the lab had not demonstrated a conclusive role for the inhibitors in the modulation of the neuroinflammatory response. Thus while the inhibitors are able to reduce neuronal death and improve behavioural outcomes it appears they are working by an as yet unknown mechanism.

4.7.5 Hippocampal area is unchanged the 5XFAD mouse

The choice to assess overall volume of the hippocampus both to extend on the previous work from Jawhar et al and also to provide another point of comparison with human studies where hippocampal atrophy is a defining characteristic of AD (Frankó et al. 2013) . As expected, hippocampal volume increased with age, however a difference in hippocampal volume between the wild type and 5xFAD mice was not noted at any age, in line with previous work demonstrating no neuronal loss in the hippocampus (Jawhar et al. 2012, Saul et al. 2013). Thus there appears to be no overt neuronal or volume loss within the 5xFAD hippocampus, in turn suggesting that the model would not be appropriate for assessing this aspect of IRAP-I function.

While the 5xFAD model demonstrates behavioural deficits, particularly pronounced beyond 10 months of age (Devi et al. 2010), this work argues that these memory deficits do not result from overall neuronal body or volume loss, but rather may be caused by synaptic degeneration or neuronal dysfunction. Decreased levels of synaptophysin have been reported in the 5xFAD model (Oakley et al. 2006, Fragkouli et al. 2014) as well as a measureable decrease in the number of dendritic spines in the stratum lacunosum moleculare of the hippocampus Neuman et al. (2015)..

4.7.6 Performance in memory tasks by 5xFAD mice treated with HFI-419

Despite the lack of effect seen on plaque deposition in the IRAP KO mice crossed with the 5xFAD animals there was still interest as the potential efficacy of treatment with the IRAP-Is. As the IRAP KO animals themselves display cognitive deficits (Yeatman et al. 2016) it would not have been feasible to investigate cognition in these animals, however there may be potential for IRAP-

I treatment to rescue the deficits induced by the AD plaque pathology. While inhibitor treatment did not result in the expected enhancement of cognition there are mitigating factors to consider. In the NOR and y-maze assessing spatial working memory both the wild-type and 5xFAD animals performed at chance levels (50%). While this would be expected of the transgenic animals as it indicates an impairment of memory, the wild-type animals should have been able to successfully remember the familiar object in the case of the NOR as well the sequence of arm entries in the y-maze. This study used an inter-trial-interval of 30 minutes, which has been demonstrated to be appropriate for discriminating between wild-type and 5xFAD animals (Joyashiki et al. 2011, Tohda et al. 2011). This suggests that rather than the wild-type mice also having an impairment of memory, it is possible that the testing conditions may not have been optimised for our testing facilities, or that the selection of objects/spatial cues made the test too hard. As such, it cannot be concluded that transgenic mice were reliably displaying a memory deficit on these tasks and following from this the efficacy of the HFI-419 treatment of rescuing/enhancing memory. Paralleling this in the spatial recognition test, both wild-type and transgenic animals performed above chance to an equal level. This means that this task may have been too easy such that even the supposedly cognitively impaired 5xFAD animals were able to complete it and indeed the animals may have reached a ceiling of performance on the task meaning that HFI-419 treatment was unable to promote further increases in memory. The choice to use a 1-way ANOVA as opposed to singular comparison between the treated groups was made for this reason, in order to be able to detect a deficit in memory in the transgenic animals and whether this could be rescued in the treated group.

The 5xFAD mice were examined at 26 weeks of age (~6.5 months old) here, an age at which several memory deficits are apparent within the model (Kimura et al. 2009, Urano et al. 2010) making it likely that lack of effect seen on memory was not due to an inherent property of the animals and once again highlighting the complexity of behavioural memory testing. Following from the work and complications discussed in the previous chapter, particularly the bell-shaped dose response curve of the IRAP-Is combined with the difficulties in performing behavioural tasks, this highlights the importance of having cellular

markers to assess in order to judge inhibitor efficacy. As before, quantification of dendritic spines provides a reliable method to assess the potential cognitive enhancing properties of IRAP-Is even in the face of these testing issues. Further, this work will be the first to assess the impact of IRAP-I treatment on dendritic spine density *in vivo.*

4.7.7 Reduction in spine density in the 5xFAD Alzheimer's mouse model

Given the ability of the IRAP-Is to increase dendritic spine density in culture there was particular interest as to whether this effect would be replicated *in vivo,* particularly in a disease state such as Alzheimer's. However, as with the hypometabolism there is debate within the literature as to precise phenotype of the AD mouse models regarding dendritic spine density making important also to characterise this aspect of the mouse models.

When taken broadly, previous work does suggest that there is a reduction in the density of dendritic spines in the Alzheimer's mouse models, with several studies demonstrating a reduction in hippocampal spines with disease progression in models such as the Tg2576 (Jacobsen et al. 2006), PSAPP (Smith et al. 2009) and 3xTg (Bittner et al. 2010). However while both Jacobsen and Smith both reportedly show spine reductions from as early as 4 months of age, Bittner and colleagues noted normal spine densities up to 10 months of age with reductions only seen in 15 – 20 month old animals. Earlier work in the PSAPP model also demonstrated no reduction at 12 months of age (Takeuchi et al. 2000). Indeed there is even work to suggest the opposite, with both the Tg2576 and PDAPP models noted to have early life reductions that returned to normal with advancing age (Lanz et al. 2003).

When looking to the literature regarding the 5xFAD model specifically, reductions in in layer V of the cortex have been noted (Buskila et al. 2013, Crowe et al. 2014) and mice between the ages of 11 – 15 months of age did show reduced spines in the stratum lacunosum-moleculare, however there were no reductions within the hippocampus proper (Neuman et al. 2015). The current work demonstrated that 6-month-old animals show a reduction in dendritic spine density within the CA1 region, similar to human condition and at an age where behavioural deficits are noted (Urano et al. 2010, Jawhar et al. 2012).

Why the animals display spine loss and not reductions in CGU could suggest that the processes are differentially affected by the disease pathology, spines being particularly vulnerable to the toxic effect of the Aβ plaques and oligomers (Wei et al. 2010, Wu et al. 2010), while another factor may be required to cause the hypometabolic phenotype. Overall, this is a particularly important finding as the beneficial effects of IRAP-I on cognitive enhancement are likely linked to their ability to enhance dendritic spine density, thus with the 5xFAD model replicating this feature of the human condition there is still space for investigation of the efficacy of the IRAP-Is.

4.7.8 IRAP inhibitor treatment increases spine density in vivo

By far the most promising work from this study is the demonstration of the ability of HFI-419 to enhance dendritic spine density *in vivo* with chronic treatment, both in wild-type animals and also the 5xFAD model. With work already demonstrating the cognitive enhancing potential of the IRAP-Is (Lee et al. 2004, Albiston et al. 2008) and their ability to enhance spines in culture, both in published work (Diwakarla et al. 2016a, Diwakarla et al. 2016b) and from the previous chapter, this result cements the cognitive enhancing capacity of the IRAP-Is by showing enhanced spine density in an animal model. The ability to rescue and possibly even enhance dendritic spine density in the 5xFAD mouse is also highly relevant given that dendritic spines are reduced in the human AD (Arendt 2001). This places the IRAP-Is as potentially being able to not only treat the symptomatic memory loss, but also the underlying cause of the loss of synaptic plasticity.

Another important aspect that these results show is the ability of HFI-419 to enhance dendritic spine density when administered peripherally. To this point IRAP-Is have been delivered directly into the brain, whether they are the peptide inhibitors (Wright et al. 1993) or the synthetic inhibitors (Albiston et al. 2008). The work here, particularly in the wild-type animals, shows that peripherally administered HFI-419 can enhance dendritic spine density, paving the way for further investigation as to its memory enhancing properties and supporting its classification as a true nootropic agent.

4.7.9 Potential of IRAP-Is in the treatment of AD

Overall, considering the phenotype of the 5xFAD mice, drawing conclusions about the efficacy of the IRAP-Is in the treatment of AD is difficult to determine. Considering the enhancement of spine density by inhibitor treatment, this would appear to suggest that the IRAP-Is could serve as successful therapeutics in the treatment of AD. However, while spine loss is a pathological feature of AD and likely contributes to the cognitive deficits observed in the disease, it is important to remember that the spine loss itself is caused by an underlying factor, be it amyloid plaques or otherwise. In this sense, the inhibitors would only function as a symptomatic treatment, similar to the currently available cholinesterase inhibitors. While there is undoubtedly benefit in having a therapeutic that targets a separate mechanism and the efficacy of the IRAP-Is could be greater than the currently available options, the real need lies in a treatment for the pathology itself.

This highlights the need to accurately phenotype the AD mouse, both to determine the potential efficacy of the IRAP-Is but also all potential AD therapeutics. Hypometabolism plays a key role in the development of AD in the human condition and given the interaction of IRAP with glucose uptake this represented a possible mechanism of action for treating the disease. However, the 5xFAD mouse did not display cerebral hypometabolism making it difficult to know when the IRAP-Is can indeed modulate the underlying pathology behind the disease. In a similar vein, there was no observed hippocampal atrophy, nor was there the expected link between plaque coverage and inflammation. Thus while the IRAP knockout did not alter plaque coverage for example, without a model that shows all of the key characteristics of AD, such as the hypometabolism, it is difficult to come to a conclusion as to the potential efficacy of the IRAP-Is in the disease. As mentioned earlier, hyper phosphorylated tau may interact with glucose transporters and therefor with the action of the IRAP-Is. However, the triple transgenic mouse models also do not display cerebral hypometabolism in a similar manner to the human condition (Nicholson et al. 2010, Ye et al. 2016). This means that testing the IRAP-Is in these models may not answer the question as to whether tau would impede their action or whether the IRAP-Is would overcome the deficit induced.

4.8 Conclusion

This study sought to provide a proof-of-concept for the effectiveness of targeting IRAP as a potential therapeutic for Alzheimer's disease. A pilot study examining the IRAP KO crossed with the 5xFAD mouse did not find any effect on plaque coverage and this necessitated the phenotyping of the model to assess its validity in examining the IRAP-Is. This work found no differences in cerebral glucose uptake or hippocampal atrophy from 2 to 18 months of age and inflammation did not appear to correlate with plaque load. Importantly however, a reduction in spine density in the CA1 region of the hippocampus was noted, mirroring one of the most important aspects of the human condition. This work also demonstrated that treatment with HFI-419 is not able to enhance dendritic spine density *in vivo* but that it is able to rescue the loss observed in the 5xFAD mouse. This places the IRAP-Is not only as a potential symptomatic treatment for the cognitive effects of AD, but also the underlying dendritic spine pathology and also as a true nootropic agent, expanding their scope of use to other disease pathologies.

Chapter 5

Summary and Future Directions

As outlined in earlier chapters, aside from memantine and the cholinesterase inhibitors there is a distinct lack of effective cognitive enhancing agents. This is particularly from the perspective of dementia, but also more broadly when considering other diseases associated with cognitive impairment such as stroke and traumatic brain injury. Although the cholinesterase inhibitors and memantine are currently prescribed in the treatment of AD and do have positive effects on the cognitive symptoms of the disease, their efficacy is limited (Casey et al. 2010). While there have been many promising targets for drug development with demonstrated efficacy in animal models, none have yet made it to market. Our lab has focused on the protein IRAP and have developed a family of synthetic inhibitors, already shown to enhance memory in rodents (Albiston et al. 2008), however the mechanism still remains elusive. Given the varied suggested characteristics and functions of IRAP, including; cleavage of various peptides, antigen cross presentation, as well as its interaction with GLUT4 and also taking into account the wide distribution of IRAP throughout the body (Thomas et al. 2003), this is not surprising. In order to narrow down the possibilities we require a more definitive understanding of the paradigms in which we assess the IRAP-Is. This concept formed the core of the current work and drove an investigation of three paradigms; behavioral memory testing, primary hippocampal cell cultures and the transgenic Alzheimer's mouse models.

5.1.1 IRAP-Is are true cognitive enhancers

Much of the previous work using both the peptide inhibitors of IRAP, as well as the newer synthetic inhibitors, has been conducted in rats that receive intracerebroventricular drug administration via a chronic indwelling cannula. While the claims resulting from this are that these drugs are cognitive enhancers, there remains the possibility that they are correcting a deficit in memory induced by the surgery procedure itself. While these results would still be valuable, they would not aid in the development of novel cognitive enhancers. Previous studies have shown an inflammatory response and impairment in memory in response to device implantation within the brain (Frumberg et al. 2007, Hirshler et al. 2010). This distinction is important to make, compounds

with an anti-inflammatory action could appear to enhance cognition as they correct the deficit, however once they were tested in a setting that did not induce neuroinflammation, such as following peripheral administration, they would likely prove to be ineffective. The work here demonstrated that while cannulation into the lateral ventricle produced a localised immune cell response, this was spatially restricted around the cannulation site and did not prompt an upregulation of inflammatory cytokines post-surgery. As the analgesic agent used here, meloxicam, has an anti-inflammatory property this could have contributed to the lack of upregulation. As this study was designed to test the surgery procedure as a whole against naïve rats, this still supports the ability of the IRAP-Is to enhance normal memory as opposed to rescuing a deficit.

While there was a slight impairment to motor coordination, the cannulation did not impair either recognition or spatial memory, indicating that agents delivered via this route, particularly the IRAP-Is, are enhancing normal memory. While this does not mean to discount compounds with potential neuroprotective effects, it seeks to highlight the unique role that the IRAP-I's may play as true nootropics. As mentioned earlier, a comparison between vehicle treated and surgically naïve animals would beneficial, however as the drug load delivered in IRAP-I studies is very small it is unlikely that this would alter the conclusions of the current work.

5.1.2 Future directions: IRAP-Is as nootropics

While there are many compounds currently used off-label as potential nootropics, there is not only concern regarding the safety of these compounds, but also substantial conflicting evidence as to their potential efficacy (Repantis et al. 2010, Ragan et al. 2013). Further, currently prescribed drugs such as donepezil (a cholinesterase inhibitor used in the treatment of AD) have reports of impairing cognition in healthy subjects (Beglinger et al. 2004, Beglinger et al. 2005), likely due to perturbation of a finely tuned cholinergic system. This highlights the importance of the IRAP–Is ability to enhance normal cognition, which sets them aside from the existing nootropic agents. Additional evidence for their potential efficacy is in the fact that they have already been shown to enhance performance in a broad range of memory domains including;

- Passive and active avoidance (Braszko et al. 1988, Braszko et al. 1991, Wright et al. 1993) tasks which have amygdala involvement (Slotnick 1973)
- Novel object recognition tasks (Paris et al. 2013), which involve portions of the frontal cortex (Antunes et al. 2011)

And spatial memory tasks which are largely hippocampal dependent such as the;

- Morris water maze (Wright et al. 1999, Olson et al. 2004)
- Radial arm maze (Olson et al. 2010)
- Barnes circular maze (Lee et al. 2004)
- Spontaneous alternation task (Albiston et al. 2008, De Bundel et al. 2009)

IRAP is also highly expressed in these areas of the brain across species (Moeller et al. 1995, Moeller et al. 1996, Chai et al. 2000) lending further support to its abilities of multi-domain cognitive enhancement. There is the potential for the IRAP-Is to be used as cognitive enhancers for students or those in cognitively demanding environments, although there are significant ethical questions to consider along this path, particularly regarding unequal access and cheating (Ragan et al. 2013, Singh et al. 2013). Less controversial is the potential for the IRAP-Is to be used in a wide range of cognitive disorders. While drugs such as donepezil are able to boost an impaired cholinergic system, as mentioned this may not be effective in other paradigms, possibly restricting their use. In contrast, the IRAP-Is can act as cognitive enhancers in a broad range of disease states. In AD for example, there is a decline in episodic memory (Irish et al. 2014) and dendritic spine density (Ji et al. 2003), a feature common to stroke pathology (Brown et al. 2008) and a parameter that the IRAP-Is have been demonstrated to positively modulate in this study. Moving forward, demonstration of the inhibitors to enhance cognition in a variety of experimental disease states as well as in high order animals in more complex tasks would be a great step towards providing additional evidence for possible efficacy at enhancing cognition in human trials.

5.1.3 Mechanisms of IRAP-I mediated cognitive enhancement: role of glucose uptake

The next stage of work aimed to determine a mechanism for this enhancement of memory while also developing a sensitive *in vitro* bioassay to screen for novel IRAP-Is. As highlighted in this work, administering IRAP-Is to rodents and performing behavioral memory assessments is a lengthy process compounded further by the tight bell-shaped dose response curve the inhibitors display (Benoist et al. 2011, Diwakarla et al. 2016a). Thus there was a pressing need for an *in vitro* bioassay that can screen for novel compounds with cognitive enhancing properties.

Dendritic spines were chosen as a marker here as they form the building blocks of memory, with their density correlating directly with cognitive status (Mi et al. 2017), and IRAP-Is have already been demonstrated to enhance dendritic spine density (Benoist et al. 2011, Diwakarla et al. 2016a, Diwakarla et al. 2016b). This work expanded on these findings by demonstrating that IRAP-Is appear to preferentially enhance the growth of mushroom spines, the morphology associated with the storage of memory and supporting the cognitive enhancing potential of the IRAP-Is. It also demonstrated cognitive enhancement and increased dendritic spine growth with the same inhibitor for the first time. Furthermore, it identified a key mechanism underlying this spine growth as increased glucose uptake through GLUT4.

While the dendritic spine assay is far more rapid than behavioral testing it is still quite time consuming, owing to the need for experimenters to manually count the spines. While not a concern in isolation, when considering the large family of potential synthetic IRAP-Is, not only benzopyran derivatives but also the aryl sulfonamides, the need for an assay capable of screening a large number of compounds becomes apparent. The measurement of radiolabelled deoxy-glucose uptake assay in hippocampal neuron cultures will now inform which compounds are likely to be successful at enhancing dendritic spine density and in turn inform which are likely to go on to produce cognitive enhancement.

This work showed that inhibition of glucose uptake by indinavir was able to abolish the IRAP-I mediated increase in dendritic spine growth. What is

particularly interesting about these results is that the bell shaped dose response was observed in both the dendritic spine *and* the glucose assay. As mentioned, the bell shaped dose response is a common feature of many nootropics (Stoiljkovic et al. 2015), however, the reasons for it vary from compound to compound. In the case of those that promote increased attention (usually stimulants) the Yerkes-Dodson law comes into effect (Figure 5-1). This describes the relationship between arousal and performance on cognitive tasks where small increases in arousal increase attention and interest in the task, while further stimulation beyond impairs performance due to anxiety.

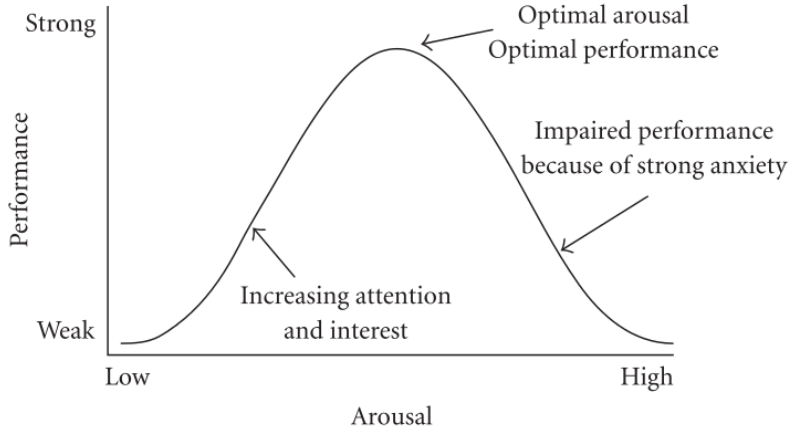


Figure 5-1 Yerkes-Dodson law, the effect of arousal on performance in memory tasks

As arousal increases, attention, interest and subsequently performance on cognitive and mechanical tasks increases. However, over stimulation leads to impaired performance as due the interference of anxiety

In the case of compounds such as the racetams, which are predicted to increase acetylcholine (Winblad 2005), a similar bell shaped curve is observed. In this case, small increases in ACh release would facilitate memory formation, however too large of an increase would lead to over excitation of neuronal cells, impairing memory and at worst lead to excitotoxicity. The IRAP-Is have also been demonstrated to be able increase the release of acetylcholine during neuronal depolarization (Lee et al. 2001), however the inhibitors themselves

cause a bell shaped dose response in the release of acetylcholine, rather than promoting ever greater amounts of acetylcholine to be released which would lead to over excitation.

In this work with the IRAP-Is, it was initially predicted that the cognitive dose response curve was due to the glucose uptake. Using mTOR as an example, small increases in glucose uptake in neurons would promote increased mTOR activity that would facilitate the growth of dendritic spines. However, large uptake of glucose would result in over activation of mTOR and could also lead to mitochondrial hyperactivity (Morita et al. 2015) that would impair neuronal function and in turn impair memory formation. However, as demonstrated here, the IRAP-Is display a bell shaped dose response in terms of glucose uptake itself. This means that there is an intrinsic property of the IRAP-Is that affects their mechanism. Off target effects at higher doses are unlikely explanation. The dose response curve is extremely narrow with only a single concentration proving to be significant in the case of both HFI-419 and SJM-164. This effect was seen in both the glucose and spine assays and combined with the relative selectivity of the inhibitors for IRAP make it more likely that there is some intrinsic factor that contributes the shape of the curve.

Speaking to the glucose assay itself, the need for it is exemplified by the results seen to SJM-164 and VR-006. Although the relatively low group numbers and variability in the performance of the control animals must be taken into account, this work was unable to demonstrate an increase in performance on the spontaneous alternation task with both compounds, however SJM-164 was able to enhance spine growth and glucose uptake. While SJM-164 was administered at the same effective dose as HFI-419 in the memory tasks, as well as a five-fold higher dose to account for the lower affinity we cannot be sure of the bioavailability of the compound even when delivered directly into the ventricle. Combined again with the tight response curve means that even small variations in how the compounds are metabolized could alter the effective dose reaching IRAP at the target site. This highlights the utility of screening novel compounds for selection to prior to progressing to cognitive testing. While the dendritic spine assay is valuable here, it is not suitable for high-throughput

screening owing to the need for the experimenter to manually count to number of spines. Thus, the glucose uptake assay forms a far more rapid *in vitro* assay for the assessment of novel IRAP-Is with potential cognitive enhancing properties.

5.1.4 Future Directions: Mechanisms of cognitive enhancement

Currently the factor that most hinders *in vivo* research of the IRAP-Is is identification of the precise mechanism governing this bell-shaped dose response. Among those that use off-label drugs as nootropics, one of the most common discussion points is how to design dosing to find the most effective doses in each of the compounds. Identification of the mechanism causing this with the IRAP-Is could drive development of compounds that could potentially avoid the fall in cognitive enhancing efficacy with higher doses, or identify possible compounds that could be co-administered with the IRAP-Is to offset the deficit. Co-administration of two compounds with separate mechanisms of action would allow for greater submaximal dosing, in turn increasing the dosing range, particularly relevant as the compounds move towards clinical applications.

Another key point in the development of the IRAP-Is will be clarification that the glucose uptake that is promoting the increase in spine density is the same as that promoting an increase in memory and learning, however to reiterate from chapter three, there are two major caveats to be discussed. The first regards the effect of the IRAP-Is *in vivo*. Speaking briefly to glucose dynamics during behavioral tasks, as animals perform a task there is a decrease in extracellular glucose levels in the hippocampus, implying uptake into neurons. A previous report suggested that administration of IRAP-Is did not cause a further decrease in glucose levels in the extracellular fluid, implying that increased neuronal glucose uptake was not occurring (De Bundel et al. 2009), however it is possible that during periods of increased neuronal glucose uptake there may be a shift towards astrocytes utilizing lactate as a source of fuel, thus the overall extracellular pool of glucose would remain constant (Simpson et al. 2007).

The second point concerns the timing of effects. As noted in the third chapter, single dose administration of the IRAP-Is did not induce changes in spine density, however cognitive enhancement is reported five minutes post administration in the spontaneous alternation task. This suggests a possible two-stage effect of the inhibitors, the initial increase in glucose uptake promoting increased cholinergic release (Ragozzino et al. 1998) to improve performance on working memory tasks while also activating downstream pathways such as the mTOR cascade.

Most importantly, the glucose uptake assay can now form a sensitive bioassay to screen for IRAP-Is with cognitive enhancing potential and will allow more rapid investigation of novel compounds. Having identified glucose uptake as the likely mechanism behind the increase in dendritic spine growth, work should now progress towards further understanding the mechanistic pathway. One of the most likely candidates is mTOR, its activation and upregulation by glucose uptake (Kimura et al. 2003, Dash et al. 2006) and subsequent effect on memory (Dash et al. 2006) and spines (Cammalleri et al. 2003) is well documented within the literature. Confirmation of this pathway could open the door toward development of different bioassays that prove to be more sensitive or rapid. Further, this will give a clearer picture as to which disease states the IRAP-Is are likely to be particularly effective in.

5.1.5 Suitability of the AD mouse model in assessing the therapeutic potential of IRAP inhibitors

With the repeated success of both the peptide (Wright et al. 1993) and synthetic inhibitors (Albiston et al. 2008) of IRAP at enhancing normal memory, as well as rescuing experimental induced deficits, such as those caused by scopolamine (Pederson et al. 1998) and alcohol (Wisniewski et al. 1993), there is great interest as to whether IRAP-Is are a potential therapeutic in the treatment of Alzheimer's Disease. The inhibitors appear to be ideally placed for this role, given that AD features cognitive decline, dendritic spine loss and glucose uptake deficits, all factors that the IRAP-Is are known to influence.

The major question at this point is whether the transgenic AD mice form a suitable model for assessing the potential of the IRAP-Is. While cognitive

decline is well noted in these models, particularly the 5xFAD used here (Ohno et al. 2006, Ohno et al. 2007, Kimura et al. 2009), whether they display dendritic spine loss and hypometabolism, particularly within the hippocampus is less clear. Speaking first to the dendritic spine density, this study demonstrated that 5-6 month old 5xFAD mice have a reduction in dendritic spine density in the CA1 region of the hippocampus and in this way the models are ideal for examining the action of the IRAP-Is as they closely mirror the human condition.

The caveat is that the current work did not find any differences in glucose uptake across age between wild-type animals and the 5xFAD. Given the pronounced and early deficits noted in humans (Mosconi et al. 2008, Mosconi et al. 2009) this lack of difference, especially considering the conflicting data within the literature, is concerning when modeling the disease. While the current transgenic AD models are invaluable in our understanding of how amyloid plaques contribute to cognitive decline, it should be acknowledged there are several limitations to the models and perhaps a better term, rather than Alzheimer's mice as is conventional, would be to label them as amyloidogenic mice.

The reason for this differentiation is pronounced when considering the interaction with IRAP. The work here did not find an effect of IRAP gene deletion on plaque load, a typical standard of assessing therapeutic efficacy. Thus there is the risk of prematurely dismissing the potential of IRAP as a novel target for AD therapy. However, as the likely mechanism of IRAP-I action is to increase glucose uptake, it is crucial that any deficit model that it is assessed in displays the human phenotype of a reduction in cerebral glucose uptake, particularly in key areas of memory and learning such as the limbic system. In addition to causing symptoms in its own right, reduced glucose metabolism may exacerbate amyloid deposition. Induction of energy deprivation has been noted to increase the levels of Bace1 (an amylodiogenic enzyme) and also increase the cerebral levels of toxic amyloid fragments such as Aβ40 in Tg2576 mice (Velliquette et al. 2005). It has also been suggested that deficits in glucose uptake lead to an increase in oxidative stress within neurons that can alter amyloid processing and exacerbate toxic amyloid production (Mosconi et al. 2008). While not

suggesting that hypometabolism is *causative* of AD, the fact that 5xFAD did not appear to display hypometabolism could explain why no effect on plaque pathology was noted with the IRAP KO mice. The most accurate conclusion to draw from this would be that the IRAP-Is likely do not affect plaque deposition or clearance in an amyloid based model, however as mentioned previously, they are able to positively affect dendritic spine density.

One of the major questions to then be asked is whether there is a suitable Alzheimer's model in which to assess the IRAP-Is. The 5xFAD model examined in this thesis in an amyloid only model, as are the vast majority of those used in the literature, however there are other AD models. The triple transgenic 3xTg mouse also features the human tau protein, thus they mirror the pathological features of the human condition more closely, yet they still do not display the characteristic hypometabolism (Nicholson et al. 2010, Ye et al. 2016). In the context of the IRAP-Is this makes evaluation of their potential efficacy more difficult. One of the possibilities discussed earlier was the down regulation of the GLUTs by tau and whether this would negatively effect IRAP-I action, or whether IRAP-I treatment would be to overcome this deficit an serve as a treatment.

Another AD model used lately in the literature is one induced by intraventricular injection of streptozotocin (Grieb 2016). In brief AD has been speculated to be "Type III diabetes" due to the hypometabolism present within the brain. Following this logic it has been suggested that this "brain diabetes" can be induced centrally in the same manner as peripherally. The major caveat to this theory is that when administered peripherally, strepzotocin induces diabetes through the destruction of beta cells in the islet of langerhan, thereby reducing insulin secretion. This is due to streptozotocin being taken up into beta cells due to its preferential transport by GLUT2, however the levels of GLUT2 in the brain are relatively low and normally found in specific regions such as the hypothalamus, while expression within the hippocampus is unclear and low at best (Arluison et al. 2004). Further, the reduction of glucose metabolism in the neocortex and hippocampus can be reproduced by other cytotoxins such as p-chlofoamphetamine (García-García et al. 2015). In summary, there does not

currently appear to be an adequate AD model in which to assess the IRAP-Is demonstrating the need for further research more broadly.

5.1.6 Future Directions: IRAP-Is as potential therapeutics

While the transgenic AD mouse models may not be completely ideal for assessing the IRAP-Is, there are other potential pathologies that warrant further investigation. As discussed earlier in the piece, reduced cerebral glucose metabolism is a common feature of several brain disorders, such as Parkinson's (González-Redondo et al. 2014, Firbank et al. 2017), and raises the possibility of using IRAP-Is as a potential therapeutic in these cases.

Looking more broadly to other disease states, ischemic stroke stands out as a possibility. In addition to producing cognitive deficits and a loss of dendritic spines (Brown et al. 2008), it is also associated with a reduction in cerebral glucose metabolism, an effect noted in; humans (Baron et al. 1986, Binkofski et al. 1996), rodents (Shiraishi et al. 1989) and primates (Touzani et al. 1995). Indeed, data from our lab has already demonstrated that IRAP knockout mice display favorable outcomes following stroke (Pham et al. 2012) and both HFI-419 and SJM-164 have reduced infarct and behavioral deficits post-stroke (*Telianidis, unpublished)*. In light of this, the possible role of IRAP-I action of glucose uptake as the possible mediating factor for these improvements deserves further investigation.

With the discussion of any drug treatment there is the necessary need to recognize potential adverse effects. While the IRAP KO models have been associated with learning and memory deficits in later life it is likely that this is due to the absence of IRAP in the developing brain and would likely not be replicated by the inhibitors. Indeed, long-term treatment of animals has not been associated within any adverse effects either in this study or by collaborators. There is the potential that the biphasic bell shaped dose response could be due to a detrimental effect of the higher doses of the inhibitor, highlighting the need to fully understand IRAPs function within the body.

Most important however are the results that demonstrate IRAP-I mediated enhancement of dendritic spine density, even in wild type animals. This confirms that the IRAP-Is are true cognitive enhancers and at the very least

could function as an adjunct therapy to treat memory problems associated with a wide variety of conditions. Currently there are no approved medications that can enhance memory and learning, and though several are used off-label, such as Modafinil and Ritalin, the efficacy and particularly, safety, of these compounds is not well defined. Further, in the case of both of the mentioned drugs, both act to enhance attention and concentration rather than directly interacting with memory formation itself. This work, through the *in vitro* hippocampal neurons and with the results seen *in vivo* in both wild-type and transgenic mice cements the IRAP-Is ability to directly influence the substrates of memory itself, dendritic spines.

5.2 Conclusion

Currently there are few true nootropic agents on the market, with those that are currently available only effective in deficit states, or often enhancing attention and focus rather than memory itself. By demonstrating that the i.c.v surgery used to assess the IRAP-Is does not impair memory I have demonstrated that there is promise for the IRAP-Is in this regard, given that they are able enhance normal memory rather than rescue a deficit state. This was expanded upon by the work demonstrating the increase of dendritic spine density in response to treatment, in particular a preferential increase in mushroom spine density. This shows that IRAP-Is are able to make lasting changes to neurons and directly affect the cellular components of memory. Further, there is now evidence that this increase is a result of enhanced glucose uptake, a factor that now allows for a rapid *in vitro* bioassay to screen for novel cognitive enhancing compounds.

The work of the final chapter demonstrated that there are several key phenotypic differences between the 5xFAD mouse model and that of human Alzheimer's, particularly in regards to cerebral glucose metabolism. While this likely precludes them from use to assess the efficacy of the IRAP-Is in the disease overall they still modeled the loss of dendritic spine. From this came the most important work of the thesis. Treatment of animals with HFI-419 demonstrated for the first time that inhibition of IRAP is able to enhance dendritic spine density *in vivo,* not only in a disease state but also in the wild-type animals,

demonstrating its ability to enhance spines when delivered peripherally but also cementing its ability to enhance normal cognition. These results support the continued research into the IRAP-Is with the goal of developing true nootropic agents.

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APPENDIX

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Research article

Cannula implantation into the lateral ventricle does not adversely affect recognition or spatial working memory

Benjamin Seyer[a], Vi Pham[a], Anthony L. Albiston[b], Siew Yeen Chai[a,*]

[a] Biomedicine Discovery Institute, Department of Physiology, Faculty of Biomedical and Psychological Sciences, Monash University, Clayton, VIC 3800, Australia

[b] College of Health and Biomedicine, Victoria University, St. Albans Campus, Melbourne, VIC 8001, Australia

HIGHLIGHTS

- Previous work suggests cranial cannulation can impair memory.
- Here we demonstrate that cannulation into the ventricle does not impair recognition or spatial memory.
- Further, this surgery induces only minor inflammatory damage that is localized to the cannulation site.

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ABSTRACT

Indwelling cannulas are often used to deliver pharmacological agents into the lateral ventricles of the brain to study their effects on memory and learning, yet little is known about the possible adverse effects of the cannulation itself. In this study, the effect of implanting an indwelling cannula into the right lateral ventricle was examined with respect to cognitive function and tissue damage in rats. Specifically, the cannula passed through sections of the primary motor (M1) and somatosensory hind limb (S1HL) cortices. One week following implantation, rats were impaired on the rotarod task, implying a deficit in fine motor control, likely caused by the passage of the cannula through the aforementioned cortical regions. Importantly, neither spatial working nor recognition memory was adversely affected. Histological examination showed immune cell activation only in the area immediately surrounding the cannulation site and not spreading to other brain regions. Both GFAP and CD-11b mRNA expression was elevated in the area immediately surrounding the cannulation site, but not in the contralateral hemisphere or the hippocampus. Neither of the inflammatory cytokines, TNF-α or IL-6, were upregulated in any region. These results show that cannulation into the lateral ventricle does not impair cognition and indicates that nootropic agents delivered via this method are enhancing normal memory rather than rescuing deficits caused by the surgery procedure.

1. Introduction

Intracranial cannulations are routinely used to deliver pharmacological agents directly into the brain, both to ensure targeted action and to avoid difficulties with permeability across the blood brain barrier [1]. Compounds that are being investigated for memory and learning enhancing properties (nootropics) are commonly delivered via this route. Often the claims resulting from this are that the specific nootropic agents are able to enhance 'normal' memory function. Studies investigating rescue in a memory-deficit or brain-damage model generally involve the infusion of a compound to induce neuronal dysfunction or cell death, such as scopolamine [2] or kainic acid [3]. Given the invasive nature of intracranial cannulations it is possible that the surgical procedure itself could be causing significant damage to the brain and already causing an impaired state. Indeed, cognitive deficits resulting from both traumatic brain injury are well documented in the literature in both humans [4,5] and animal models [6] and it is possible that these effect are mediated by inflammatory cytokines given that inflammation has been associated with memory deficits [7]. In particular both TNF-α and IL-6 are upregulated following injury and have been associated with cognitive impairments [8–10].

The detrimental effects of common surgical procedures on memory function have already been demonstrated with implan-

* Corresponding author.
E-mail address: siew.chai@monash.edu (S.Y. Chai).

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tation of a metal cannula into the striatum [11] and insertion of an electrode into the subthalamic nucleus [12]. These impairments become especially relevant considering that the cellular and molecular mechanisms that govern 'normal' memory enhancement and those that are involved in rescuing a memory deficit may be completely distinct, particularly when attempting to elucidate the potential mechanisms of the nootropic agents. Thus, the present study sought to investigate the behavioural and cellular effects of an indwelling cannula implanted unilaterally into the lateral ventricle, a common route of nootropic compound delivery [1,13–15]. This route of delivery causes the cannula only to pass through the motor and somtaosenory cortices, thus it is possible that memory will not be affected. Further we sought to assess the pathological damage resulting from the surgery in terms of the level and spatial spread of immune cell activation and cytokine expression.

To assess memory, the spontaneous alternation task (SAT) and novel object recognition task (NOR) were chosen. The SAT is largely hippocampal dependant task [16] and is a measure of spatial working memory while the NOR is a measure of recognition memory and while dependent on a diverse number of brain regions also requires the hippocampus [17–19]. Both of these tasks were chosen as they are common choices for testing nootropic agents and the NOR in particular chosen to provide a direct comparison with earlier studies investigating memory in response to surgical procedures.

2. Methods

2.1. Surgery

Male Sprague Dawley rats (270–310 g, 7–8 weeks old) were housed in groups of 4 with *ad libtum* access to standard rat chow and water until time of surgery (All research conducted in this study was approved by the Monash University Animal Welfare Committee (MUAWC) under the Monash Animal Research Platform (MARP) 2011/117 application and performed according to the National Health and Medical Research Council of Australia "Code of practice for the care and use of animals for scientific purposes"). On the day of surgery, rats (n = 24) were anaesthetized with 5% (v/v) isoflurane before being placed in a Kopf stereotaxic frame and then maintained at 2% (v/v) isoflurane for the remainder of the surgery. Rats were implanted with an indwelling cannula (22 gauge from Plastics One; USA) in the right cerebral ventricle using the following flat skull coordinates; 0.8 mm posterior to Bregma, 1.5 mm lateral to the midline and 3.5 mm ventral to the surface of the skull (Fig. 1). The cannula was secured to the skull with a stainless steel screw and dental cement. During brain collection it was confirmed that the screw did not penetrate the dura. 1 mg/kg intraperitoneal Meloxicam was given during surgery and rats supplied with 15 mL of 200 mg/kg paracetamol in 10% sugar water for two nights post-surgery as analgesia. Control rats (n = 22) received no treatment or anaesthesia.

All rats (both control and cannulated) were individually housed post-surgery and handled daily for 5 min to familiarise them with human contact. All rats performed the spontaneous alternation task on day 6-post surgery and the novel recognition task on days 7 and 8. Of these animals a smaller subset completed the elevated plus maze on day 8. A second subset performed the initial rotarod task one day prior to surgery and the second trail on day 6.

Prior to each behavioural task the rats were acclimatised to the testing room for 40 min. Further, the testing apparatus in each case was sprayed and wiped down with 80% ethanol before the rat began the task to remove olfactory cues. All tests took place in the same

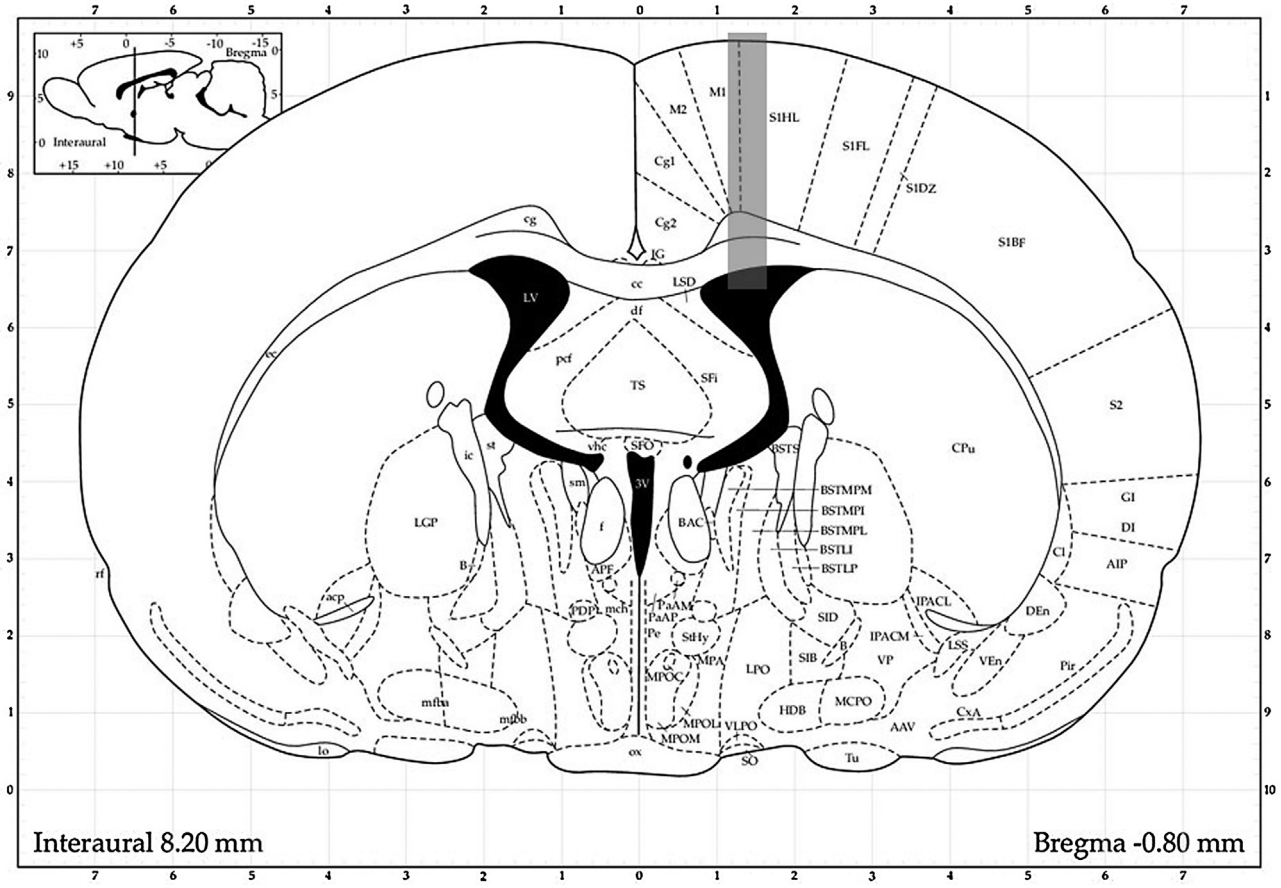

Fig. 1. Placement of the indwelling cannula into the lateral ventricle (Image adapted from "The rat brain in stereotaxic coordinates" Paxinos and Watson1982).

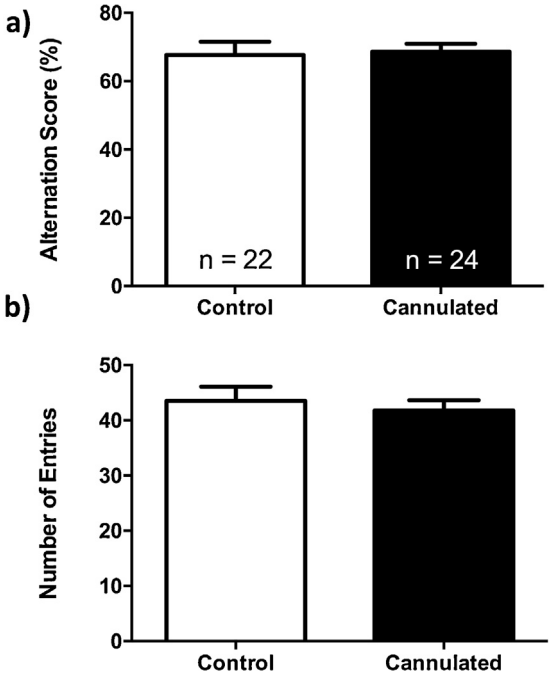

Fig. 2. Performance in the spontaneous alternation task by control and cannulated rats No difference in the alternation score between groups was seen ($p = 0.83$) over a period of 20 min in the maze. There was also no difference in the number of arm entries between either of the groups ($p = 0.58$) Data represented as mean ± SEM, P value determined by unpaired *t*-test.

room with diffuse dim lighting; the testing area itself consisted of 3 white walls and a white curtain behind which the experimenter sat during the experiments.

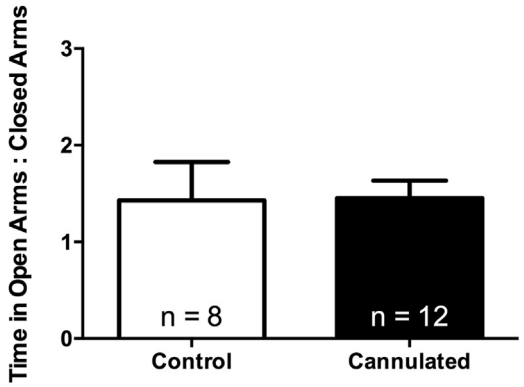

Fig. 4. Exploration of the elevated plus maze by control and cannulated rats. Both groups spent an equal amount of time in open and closed arms ($p = 0.95$) Data represented as mean ± SEM, P value determined by unpaired-test.

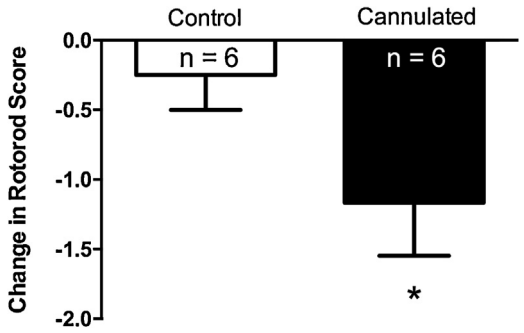

Fig. 5. Change in max running speed on the rotarod task by control and cannulated rats. All rats were able to reach the max speed of 30 rpm prior to the surgery day. When tested post surgery cannulated rats showed a greater reduction in motor coordination in the second trial compared to control animals ($p = 0.038$). Data represented as mean ± SEM, P value determined by unpaired *t*-test.

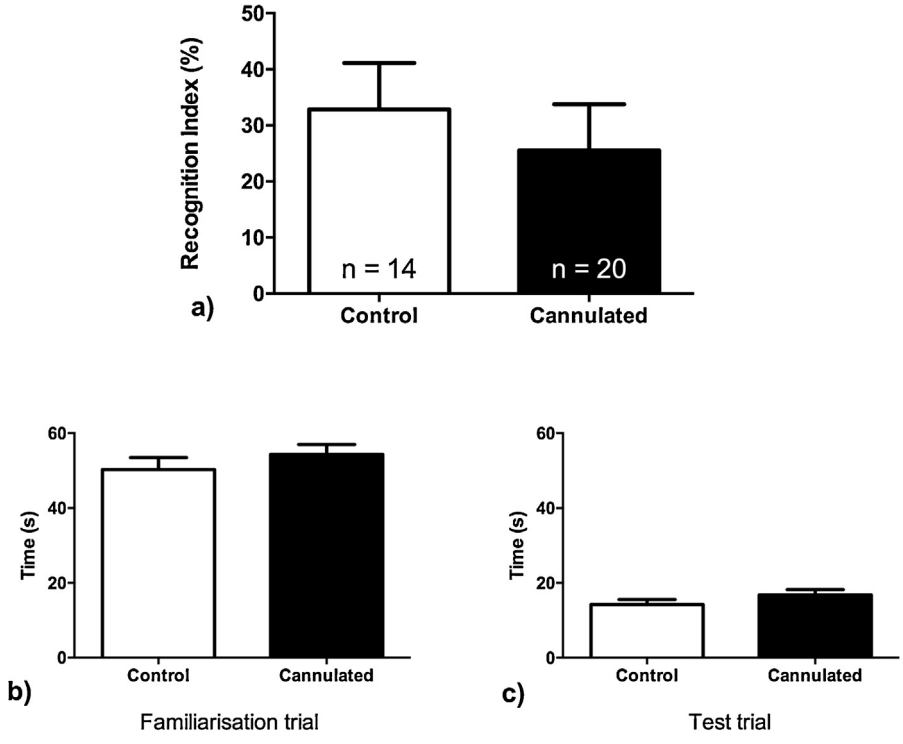

Fig. 3. Performance in the novel object recognition task by control and cannulated rats Both groups displayed a preference for exploration of the novel object, indicating they were able to recall the familiar object, though there was no difference in the recognition index between each group ($p = 0.44$). There was no difference between groups in the amount of time spent exploring the objects in either the (b) 5-min familiarisation trial ($p = 0.5$) or (c) 2-min test trial ($p = 0.22$) Data represented as mean ± SEM, P value determined by unpaired *t*-test.

2.2. Elevated plus maze

The elevated plus maze consisted of four arms 70 (long) × 10 (wide) cm with two 'open' arms opposite each other having walls of clear Perspex 5 cm high and two 'closed' arms with 27 cm high grey Perspex walls. The floors were white laminate. The maze was elevated 85 cm above the ground. Rats were placed in the central platform facing one of the open arms, and behaviour was monitored for 5 min. The ratio of time spent exploring the open arms vs. the closed arms was used as a measure of anxiety.

2.3. Spontaneous alternation task

The plus maze had four arms measuring 75 (long) × 10 (wide) × 20 (high) cm and visual cues placed 1 m away from the maze on opposites of the centre intersect. The floor of the maze was white plastic while the walls were black. The test was performed by placing the rat in the centre of the maze (all animals placed facing the same arm) and allowing 20 min of unimpeded exploration. The number and sequence of arm entries were recorded for calculation of a precent alternation score. An alternation consisted of 4 different arm choices in 5 consecutive arm entries. Dividing the number of observed alternations in overlapping quintuplets by the number of possible alternations and multiplying the quotient by 100 calculated an alternation score.

2.4. Novel object recognition task

Prior to the acquisition trial, the rats were habituated for 30 min in the testing box (made from grey Perspex of dimensions 60 × 60 × 50 cm) in diffuse dim light and then returned to their home cage for 30 min. During the familiarisation trial, rats were placed in the box facing away from two identical objects that had been secured to the floor in adjacent corners (with enough room to allow complete movement around them) of the box. Rats were allowed 5 min to explore the objects and exploration time recorded,

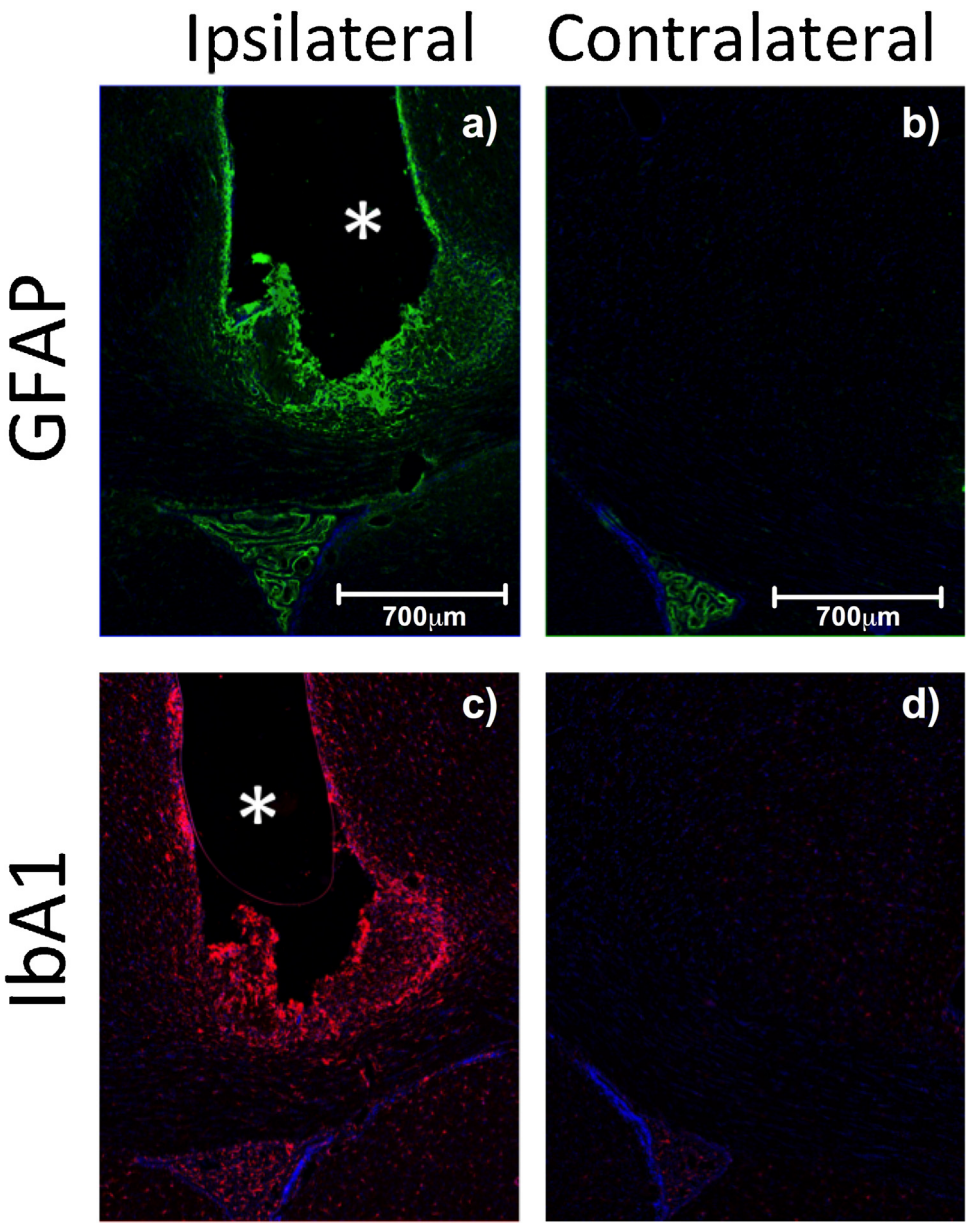

Fig. 6. Immunohistochemistry 8 days post-surgery demonstrating activated astrocytes and microglia in the lesioned cortex (a,c) and contralateral cortex (b,d) (5× Magnification). (a,b) GFAP expression shown in green highlights astrocytes, (c,d) CD-11b shown in red highlights microglia. Images representative of 3 different animals.

with exploration defined as the nose being less than 2 cm from the object when facing the object and actively engaging with it (sniffing, paw touching etc.). Climbing on the object was not considered as exploration.

After an inter-trial interval of 3 h, one of the familiar objects was replaced with a novel object made from the same material but of a different shape replaced one of the objects. During this test trial, the rats were given 2 min to explore the box. The recognition index was determined as the time spent exploring the novel object minus the time spent on the familiar object, divided by the time spent on both objects. Rats were excluded from analysis if they spent less than 30 s exploring the objects during the familiarisation trial or less than 5 s exploring the objects in the test trial. Under these criteria, 8 animals were excluded from the control group and 4 from the cannulated group.

2.5. Rotarod

Each rat was placed on a rotarod consisting of a motorised cylindrical assembly of 18 stainless steel rods capable of rotating at a maximum speed of 30 revolutions per minute (rpm) (Ratek, Australia). For the animal to maintain an upright position on the rotarod, it had to grip the rods and keep "walking" as the rods rotated. Starting at 6 rpm the speed was increased by 1.5 rpm increments every 3 s until 30 rpm was reached. In the case that a rat gripped the rods but did not walk so that it rotated with the device, or fell off the device, rotation was stopped, the speed was decreased by 1.5 rpm, the rat was replaced on top of the rotarod and rotation was resumed. Rats were given three attempts to reach maximum speed and the fastest speed at which they could maintain an upright position on the rotarod for 3 s was recorded. Each rat was tested one day prior to surgery and again 6 days post surgery and the change between these scores used to assess the level of motor impairment.

2.6. Immunohistochemistry

Following performance of the NOR (eight days post surgery) rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg) before being perfused transcardially with ice-cold phosphate buffered saline (PBS) and then ice-cold 4% (w/v) paraformaldehyde, 4% sucrose (w/v) in PBS (pH 7.3). Animals were decapitated, the cannula removed and the brains rapidly harvested before being placed in the same fixative for 24-h, cryoprotected by immersion in 30% (w/v) sucrose overnight at 4 °C and then snap frozen in isopentane on dry ice and stored at −80 °C

Twelve-micron coronal sections were cut using a cryostat and thaw mounted onto gelatin-chrom-alum coated slides. Sections were incubated with 10% (v/v) normal donkey serum in PBS with 0.3% (v/v) Triton-X-100 for 1 h at room temperature to block non-specific binding of the antisera. Sections were then incubated overnight at 4 °C with the following primary antibodies; rat anti-GFAP (Life Technologies), rabbit anti-CD-11b (Serotech) and mouse anti-NeuN (Life Technologies), all at 1:500 dilution in 3% (v/v) normal donkey serum in PBS with 0.3% (v/v) Triton-X-100. Sections were then washed three times with PBS containing 0.1% (v/v) Tween-20 and secondary antibodies applied in PBS; Alexa 488 anti-rabbit and Alexa 568 anti-rat (Introvogen). Sections were coverslipped with DAKO containing DAPI prolong gold (Life Technologies).

2.7. RT-PCR

Eight days post-surgery, rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg) until the heart just ceased beating. Eye and toe reflexes were checked to be absent before a cervical dislocation was performed, the cannula removed and the brain rapidly dissected on ice. For the cortical sample, tissue immediately surrounding the cannulation site was taken as well as tissue taken from the contralateral hemisphere in the corresponding location. The hippocampus was also dissected and divided into ipsi- and contra-lateral sides for the animals that had received surgery. For control tissue, tissue was taken from the same points although the hemispheres were collected together in this case.

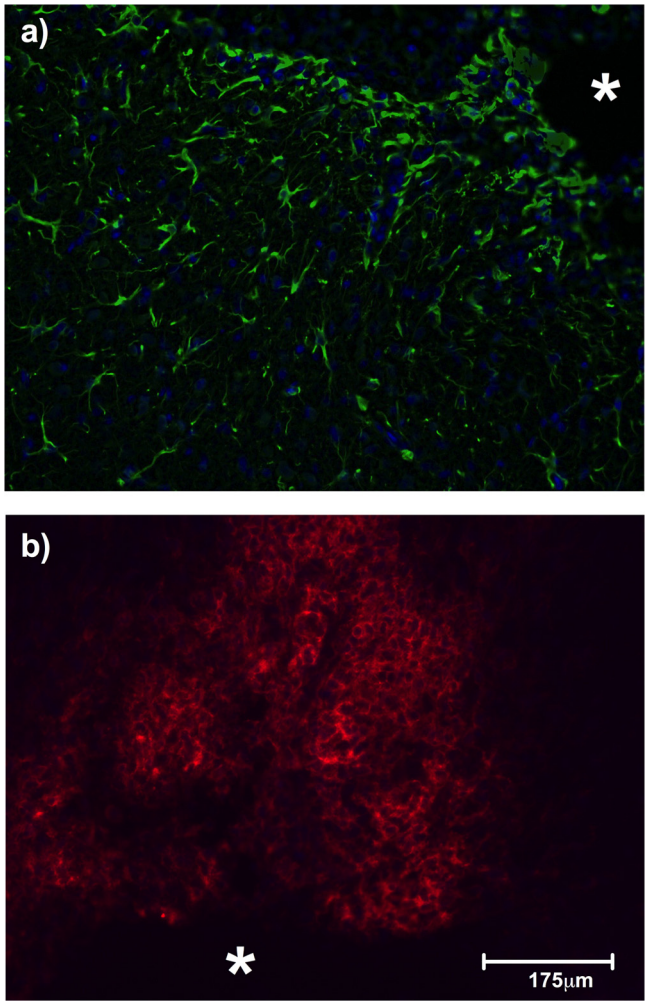

Fig. 7. Immunohistochemistry 8 days post-surgery demonstrating activated/reactive (a) astrocytes and (b) microglia surrounding the base cannulation site (cannula placement indicated by *) (20× Magnification).

Total RNA was extracted from dissected tissues (25 mg) stored at −80 °C using Trizol reagent (Life Technologies) according to the manufacturer's protocol. RNA quantity and quality were determined using A_{260}/A_{280} readings by NanoPhotometer (Implen). For RT-PCT, two microgram of RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For RT-PCR, the synthetized cDNA was used as a template for PCR reactions using TaqMan Fast Advanced Master Mix (Applied Biosystems) and appropriate TaqMan Gene Expression assay (Applied Biosystems). The TaqMan probes have the following identification number: 45 S (Rn03928990_g1), GFAP (Rn00566603_m1), CD-11b (Rn00709342_m1), TNF-α (Rn00562055_m1), and IL6 (Rn01410330_m1). Relative quantification of gene expression was performed by comparative threshold (CT) method (calculated by the $2^{-\Delta\Delta CT}$ method). Changes in mRNA expression levels were calculated following normalization to the housekeeping gene 45S.

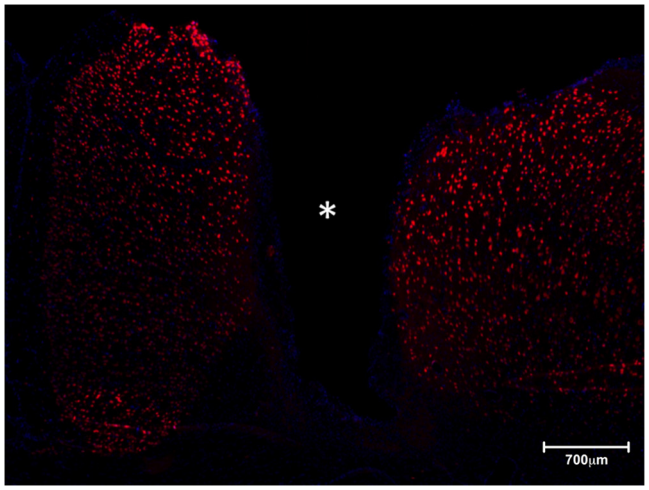

Fig. 8. Immunohistochemistry 8 days post-surgery with staining for NeuN to label live neurons.

2.8. Statistical analysis

Statistical tests performed in Grahpad Prism 6 and described under each figure as appropriate.

3. Results

We sought to evaluate the impact of a unilateral intracerebral ventricular cannulation on cognition in two separate behavioural tasks, a four-arm spontaneous alternation task to assess spatial working memory (Fig. 2) and the novel object recognition task to assess object recognition memory (Fig. 3). Each of these tasks engages brain regions beyond the hippocampus thus allowing a more definitive investigation of whether specific memory processes are affected by the cannulation. Here we saw that there was no difference in performance on either of the tasks indicating that the cannulation process does not affect memory one week post-surgery.

The cannulation surgery represents a stressor to the rats and could them to be anxious during the testing period, which would impact performance. In the elevated plus maze both groups spent an equal amount of time in the open and closed arms indicating no differences in anxiety (Fig. 4).

Considering that the cannula descended through the motor cortex before reaching the ventricle we were also concerned that the motor function of the animals could be affected. Using the accelerating rotarod we noted a slight impairment of fine motor coordination (Fig. 5). We do not believe that this adversely affected performance of the memory tasks as the number of arm entries in the SAT (Fig. 2) and the total exploration time in the NOR (Fig. 3) were the same between both groups.

Examining the damage resulting from the cannula implantation at a cellular level we noted a population of activated astrocytes and microglia at the base of the cannula, as indicated by the GFAP and CD-11b staining respectively, while there was also what we speculated to be a thin layer of scar tissue formed along the tract of the cannula insertion (indicated by the *) (Fig. 6a,c). Importantly this inflammation did spread laterally from the cannulation site and there was no evidence of inflammation on the contralateral

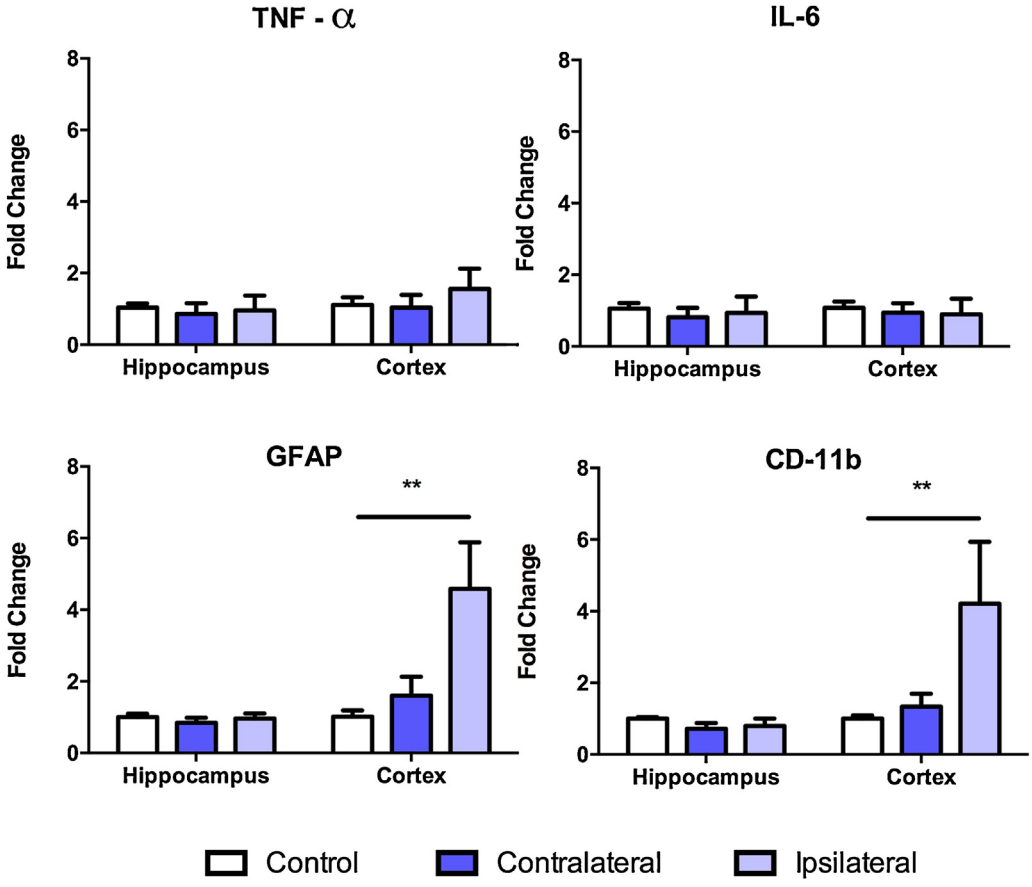

Fig. 9. mRNA expression of GFAP, CD-11b, TNF-α and IL-6 in both the cortex and hippocampus of control and cannulated rats as determined by RT-PCR. n = 5 controls and 9 cannulated animals. ** $P < 0.01$, 1-way ANOVA with post-hoc Bonferroni (separate for each cortex and hippocampus).

side of the cortex (Fig. 6b,d). The characteristic activated/reactive state of both astrocytes (as indicated by the mossy structure) and microglia (as indicated by the ameboid structure) can be observed in the higher magnification images (Fig. 7a,b) Staining for NeuN was also performed in order to visualise the extent of neuronal loss surrounding the cannula. While there is a clear area of cell death at the base of cannula corresponding to the population of activated immune cells, live neurons can be seen right up to the edge of the cannula tract (Fig. 8).

RT-PCR was also performed to assess the levels of inflammatory cytokines in the cortex and the hippocampus. Correlating well with the immunohistochemistry there was an upregulation of both GFAP and CD-11b mRNA in the area of cortex directly surrounding the cannula tract while there was no change in the contralateral hemisphere or hippocampus. No upregulation of the inflammatory cytokines TNF-α or IL-6 was noted in either cortical hemisphere or hippocampus (Fig. 9).

4. Discussion

The present study demonstrated that both spatial working memory (as assessed by the SAT) and recognition memory (as assessed by the NOR) were unaffected one week after the implantation of an indwelling cannula into the lateral ventricle (Figs. 2 and 3). This was supported by the relatively small amount of neuronal cell death (Fig. 8), limited spread of immune cell activation from the cannulation site (Fig. 6) and the lack of inflammatory gene upregulation in any area (Fig. 9).

We also addressed two areas that could have possibly confounded the study. Given the stressful nature of the surgery procedure it is possible that the rats may have been more anxious during the testing phase, however we observed no difference in anxiety (Fig. 4). While the rats did display a reduction in motor coordination (Fig. 5) this is unlikely to have affected performance on the memory tasks as indicated by the changed number of arm entries in the SAT (Fig. 2) and the same total exploration time in the NOR (Fig. 3). The deficit was likely due to the passage of the cannula through the motor cortex (Fig. 1) and the presence of localised immune cell activation with the area.

As inflammation has previously been linked to cognitive deficits we used RT-PCR to quantify the levels of immune cell and inflammatory cytokine expression. As seen in Fig. 9 (and in line with the immunohistochemistry seen in Fig. 6) there is an increase in the expression of activated astrocytes and microglia, however the absence of the upregulation of either inflammatory cytokine around the cannulation site (Fig. 9) demonstrates the relatively benign nature of the surgery. Further, it parallels evidence from both stroke [20] and trauma [21] studies demonstrating that both TNF-α and IL-6 are trending downwards or have returned to baseline levels 7 days post-insult. Both these and the histological results imply a very localised inflammatory response that does not extend beyond the immediate site of cannulation. This is supported by the lack of neuronal cell death, also in line with previous studies which have examined the cellular response to implanted devices [22]. Inflammation in humans [23], and animal models [7] have been linked to memory impairments, however these are often chronic and sustained whereas the relatively acute nature of the insult in the current study and moderate spatial spread of activated immune cells likely explains why no cognitive deficit is observed here.

4.1. Comparison with previous behavioural studies

A number of studies have examined the impact of a number of surgical procedures on brain function, however only two have explicitly examined whether cognitive processes including memory are adversely affected [11,12]. Both these studies detected impairment in recognition memory in contrast to the current study.

Both previous studies have performed pre- and post-surgery behavioural tests [11,12], while the present study only involved testing post-surgery. Pre-testing was deemed unnecessary given that the control animals did not receive either anaesthesia or surgery of any kind and reflect the animals in an unchallenged state. Secondly, testing of animals multiple testing of the same behavioural task introduces an element of learning that is avoided in this study.

The first parallel to the current study was a striatal microdialysis cannulation, which noted impairment in recognition memory as well as reduced glucose uptake in several regions including the caudate, thalamus and frontal, motor and sensory cortices [11]. The second was a bi-lateral implantation of electrodes into the subthalamic nucleus, which also caused neuroinflammatory responses in several regions of the brain including the entorhinal, parietal and frontal cortices [12]. One of the key differences between these and the current study is relative severity of the surgical procedure. The study by Frumberg and colleagues [11] descended 6 mm below the surface of the skull and deep into the caudate while Hirshler and colleagues [12] performed bi-lateral electrode insertions beginning 3 mm anterior to bregma at a 38° angle and ending 3 mm posterior, passing through prefrontal cortex, striatal areas and the thalamus before terminating in the subthalamic nucleus. In both cases, areas implicated in memory and learning may have been damaged by the cannula/electrode passage and/or subsequent inflammation. By comparison, the i.c.v cannulation used here only descended 3.5 mm into the brain and passed through motor/somatosensory cortex. Thus while direct comparison is difficult, it could be argued that fewer areas (specifically those associated with memory) are damaged by this procedure, which could account for the lack of cognitive deficit.

While the present study was designed to mimic previous work and as such did not feature volume infusion in addition to cannulation. Given that the ventricles are a fluid filled space, as opposed to a neuron dense region such as the striatum, it is unlikely that the addition of a small volume (2 μL being common [1]), would cause structural damage and adversely affect cognitive performance. However, the possibility cannot be ruled out, particularly if a constant infusion is performed, and as such future studies should be directed towards examining this.

4.2. The effect of isoflurane

While one of the more common anaesthetics used in animal surgeries, isoflurane has been linked to memory deficits in rodents [24,25], though this has been disputed by others [26,27]. The current study chose to examine the surgical procedure as a whole, rather than just the effect of the cannulation, thus the choice to not subject control animals to anaesthesia could be a point of contention. While not specifically designed to investigate the effect of anaesthesia, this work does support the notion that there is no lasting memory impairment caused by isoflurane. However this could also be due to the relatively short duration of exposure with surgery lasting 25–30 min, in contrast to most previous studies, which examined exposure times of at least 2 h.

5. Conclusion

The current study demonstrates that while an inflammatory response may be mounted following implantation of a cannula into the lateral ventricles, it appears to be spatially restricted around the site of the cannulation and is not sufficient to adversely affect cognition. This has implications for studies that seek to deliver nootropic

agents into the ventricles in order to improve memory and learning and indicates that any outcomes are due to direct positive effects on 'normal' cognition rather than rescue from a damage state.

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