2019

Exploring the Role of Insulin Receptor Signaling in Hippocampal Learning and Memory, Neuronal Calcium Dysregulation, and Glucose Metabolism

Hilaree N. Frazier
University of Kentucky, hilaree.frazier@uky.edu
Author ORCID Identifier:
https://orcid.org/0000-0003-2309-2596
Digital Object Identifier: https://doi.org/10.13023/etd.2019.298

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Hilaree N. Frazier, Student

Dr. Olivier Thibault, Major Professor

Dr. Rolf Craven, Director of Graduate Studies

Exploring the Role of Insulin Receptor Signaling in Hippocampal Learning and Memory, Neuronal Calcium Dysregulation, and Glucose Metabolism

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Hilaree N. Frazier
Lexington, Kentucky
Director: Dr. Olivier Thibault, Professor of Pharmacology and Nutritional Sciences
Lexington, Kentucky
2019

https://orcid.org/0000-0003-2309-2596

ABSTRACT OF DISSERTATION

EXPLORING THE ROLE OF INSULIN RECEPTOR SIGNALING IN HIPPOCAMPAL LEARNING AND MEMORY, NEURONAL CALCIUM DYSREGULATION, AND GLUCOSE METABOLISM

In the late 90's, emerging evidence revealed that the brain is insulin-sensitive, highlighted by broad expression of brain-specific insulin receptors and reports of circulating brain insulin. Contemporary literature robustly supports the role of insulin signaling in normal brain function and suggests that insulin-related processes diminish with aging, evidenced by decreased signaling markers, reduced insulin receptor density, and lower levels of insulin transport across the blood-brain barrier. In the context of pathological cognitive decline, clinical trials using intranasal insulin delivery have reported positive outcomes on memory and learning in patients with mild cognitive decline or early-stage Alzheimer's disease. However, while the importance of insulin and its related actions in the brain are robustly supported, the distinct mechanisms and pathways that mediate these effects remain unclear.

To address this, I conducted a series of experiments exploring the impact of insulin on memory and learning in two models: primary hippocampal cell cultures and the Fisher 344 animal model of aging. These studies attempted to identify relationships between insulin receptor signaling, neuronal gene expression, glucose metabolism, and calcium homeostasis in the hippocampus using either expression of a constitutively active human insulin receptor or administration of intranasal insulin. The following dissertation summarizes this work and provides valuable insights into the potential pathways mediating these relationships. Of note, intranasal studies reported that insulin is able to significantly alter gene expression patterns in the hippocampus of both young and aged rats following chronic, repeated exposure to the ligand. In cell culture, constitutive insulin signaling correlated with significantly elevated neuronal glucose uptake and utilization, as well as with significant alterations in the overall expression and localization of the neuron-specific glucose transporter 3. Interestingly, continued activity of the insulin receptor did not appear to alter voltage-gated calcium channels in hippocampal neurons despite prior evidence of the ligand's role in other calcium-related processes.

The results reported in this manuscript suggest that in the brain, insulin may be involved in a myriad of complex and dynamic events dependent on numerous variables,

such as age, length of the exposure, and/or the insulin formulation used. Nevertheless, this work highlights the validity of using insulin to ameliorate age-related cognitive decline and supports the need for further studies exploring alternative approaches to enhance insulin receptor signaling in the brain.

KEYWORDS: Insulin Receptor, Hippocampus, Aging, Calcium Dysregulation, Glucose Metabolism, Brain Insulin Resistance

Hilaree N. Frazier

(Name of Student)

06/12/2019

Date

EXPLORING THE ROLE OF INSULIN RECEPTOR SIGNALING IN HIPPOCAMPAL LEARNING AND MEMORY, NEURONAL CALCIUM DYSREGULATION, AND GLUCOSE METABOLISM

By
Hilaree N. Frazier

Dr. Olivier Thibault
Director of Dissertation

Dr. Rolf Craven
Director of Graduate Studies

06/12/2019
Date

DEDICATION

To my mother Anita for teaching me strength and perseverance, to my father Ralph for imparting to me his wisdom and desire to learn, and to all of my friends and family for their endless love and support.
Thank you.

ACKNOWLEDGMENTS

The work presented in the following dissertation benefited from the guidance, direction, and collaborations of several people. First, I want to thank my dissertation director Dr. Olivier Thibault for his tremendous support, guidance, and understanding throughout my nearly six years in his lab. Not only was Dr. Thibault an extremely effective advisor who oversaw my development as a student and scientist by training me on numerous laboratory techniques, but he was also an incredible mentor who provided insight into the nuances of manuscript publishing, grant writing, and networking while also allowing me ample time outside of the lab to hone my leadership and professional development skills. Additionally, Dr. Thibault, as well as all of my colleagues in the Thibault lab, provided timely and instructive feedback throughout the dissertation process, and I know that my work would not be as thorough, cohesive, or impactful if I hadn't had their help along the way.

I also wish to thank the rest of my dissertation committee, Drs. Rolf Craven, Christopher Norris, and Kenneth Fields, and my outsider examiner, Dr. Lance Johnson, for granting me their time and insight. Their comments and suggestions challenged my thinking and significantly improved the results I obtained during the course of my project.

Finally, I wish to acknowledge those in the Department of Pharmacology and Nutritional Sciences, my fellow graduate students here at the University of Kentucky College of Medicine, and all of my friends and family for their on-going encouragement and kindness. I would not be where I am today without their support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ADDITIONAL FILES

SUPPLEMENTAL TABLE 4.1 MICROARRAY ANALYSIS REPRESENTING THE PRE-STATISTICALLY FILTERED LIST OF GENES**.....**[SUPPTABLE.XLSX 2.1 MB]

CHAPTER 1. INTRODUCTION

In 1921, Drs. Banting and Best made one of the most important discoveries of contemporary medicine: the discovery of the pancreatic hormone insulin [1]. The initial purification of this ligand from depancreatized dogs eventually led to the formulation of a life-saving therapy for individuals suffering from diabetes, thus saving hundreds of thousands of lives over the past 97 years. During this time, our knowledge of insulin's physiological function has grown exponentially, and we now know that this peptide, along with its receptor, is an essential component of peripheral metabolism, mediating numerous processes including storage and synthesis of lipids, proteins, and carbohydrates, and the uptake of blood glucose from the circulation [2, 3]. Recently, the impact of this ligand has been expanded to also include important physiological functions in the brain, particularly those related to cognition.

In the following dissertation, I will demonstrate the clinical impact and biological importance of this hormone in the context of aging, cognitive decline, and hippocampal learning and memory. The following chapter will introduce the concept of the insulin sensitive brain by first providing an overview of the insulin peptide, its receptor, and the canonical insulin signaling pathway. This will then be followed by a discussion of insulin's relationship to hippocampal processes, as well as summary of therapeutic techniques designed to target impaired insulin signaling in the brain. In Chapters 2-6, I will discuss potential mechanisms mediating insulin's actions on cognitive function and present a series of related experiments I conducted during my time in the lab of Dr. Olivier Thibault. Finally, in Chapter 7, I will summarize these findings and discuss their impact and

contributions to our current knowledge regarding pathological brain aging and AD while also speculating on novel ways to utilize these findings in the future.

1.1 The Insulin Peptide

1.1.1 Pancreatic Synthesis and Secretion of Insulin

Insulin is encoded by the *INS* gene on chromosome 11 [4, 5]. Production of the hormone occurs in the pancreas, specifically in the β cells of pancreatic islets. The process begins with transcription of *INS*, resulting in the production of pre-proinsulin mRNA, which is then synthesized into the insulin precursor protein pre-proinsulin [4]. This protein is a single polypeptide containing an α chain, β chain, a connecting peptide (C-peptide), and a signal peptide. The signal peptide directs pre-proinsulin to the rough endoplasmic reticulum (ER) of the β cell where it is then cleaved and subsequently converted into proinsulin. During its time in the ER, proinsulin is folded, acquiring its three-dimensional conformation through disulfide bonds which link the α and β chains together [4]. The folded proinsulin is then transported from the rough ER to the Golgi apparatus. Proinsulin is further converted to its physiologically functional form via cleavage of C-peptide by the prohormone convertases PC1 and PC2 and the exoprotease carboxypeptidase E inside granules located in the *trans* Golgi network [4, 6]. Mature insulin is comprised of 51 amino acids (21 in the α chain and 30 in the β chain), and has a molecular weight of 5.8 kDa [4]. Inside storage granules, mature insulin crystallizes into a hexameric conformation consisting of 6 insulin monomers surrounding a central zinc ion [6]. This conformation provides stability to the protein and allows it to be stored for long periods of time yet

renders it metabolically inactive. Upon secretion, the hexamer dissociates, resulting in kinetically active insulin monomers capable of binding their receptor.

Insulin-containing granules are stored in one of two "pools:" the "rapidly releasable" pool, which remains docked and primed at the plasma membrane until signaled for release, and the "reserve" pool, which resides close by in the cytoplasm [6, 7]. Uptake of glucose into the pancreatic β-cells leads to closure of potassium channels and subsequent depolarization of the plasma membrane [7]. This depolarization opens calcium channels, allowing influx of Ca^{2+} ions into the cell. Secretion of insulin into the bloodstream is biphasic. The first phase is rapid (1-5 min) and occurs via calcium-mediated fusion of docked insulin-containing granules to the plasma membrane, while the second phase occurs more slowly (5-60 min) and involves reserve granules trafficking to this same docking site and becoming primed before fusing [6, 7]. In the periphery, secretion of insulin is primarily mediated by the level of circulating glucose, although other triggers, including amino and fatty acids, acetylcholine and pituitary hormones, and other less understood agonists, may also be involved in this process, either independently or concurrently with glucose binding [7]. Following exocytosis, insulin enters the bloodstream and eventually activates a variety of insulin-mediated physiological processes by binding to the insulin receptor (IR) in organs such as the liver and kidney. In some cases, bound insulin may dissociate from the IR after initial activation and re-enter the bloodstream, but generally, the bound ligand and its receptor are subsequently endocytosed for processing as a ligand-receptor complex [8, 9].

1.1.2 Endosomal Processing and Degradation of Insulin

The acidic environment inside the endosome triggers dissociation of the ligand from the IR. The free insulin is then processed via proteolysis, primarily by insulin degrading enzyme (IDE) [10]. Interestingly, initial endosomal degradation is usually not a complete digestion, resulting in only partially-processed insulin that is then trafficked to lysosomes for additional metabolism [10, 11]. Circulating insulin that does not bind to the IR undergoes a process similar to that of the insulin-receptor complex, including initial uptake of the ligand into the cell; however, unbound ligand is pinocytosed rather than endocytosed [11]. Further, insulin protease activity has also been detected at the plasma membrane and in the cytosol [10], suggesting that partial-processing in the endosome may not always be a required step. Prior to ligand degradation, the binding of insulin to the peripheral IR triggers the activation of signaling pathways that induce uptake of circulating glucose into adipose and muscle tissue. In the next section, I will discuss these signaling pathways in more detail, as well as provide an overview of IR synthesis, structure, and regulation in the periphery.

1.2 The Insulin Receptor

1.2.1 IR Morphology and Regulation

The mature IR is a tetrameric tyrosine kinase receptor consisting of 2 extracellular α-subunits and 2 transmembrane β-subunits [2]. Production of the peripheral IR begins with transcription of *INSR*, a 22 exon gene located on chromosome 19 in humans [10]. Following transcription, IR mRNA undergoes alternative splicing to generate one of two

variants: the IR-B sequence that includes all 22 exons, or the IR-A sequence that does not possess exon 11 [12, 13]. Translation of these variants produces either IR-A or IR-B isometric monomer peptides comprised of 8 distinct domains which then self-associate via disulfide bonds to form a proreceptor [10]. During this process, monomers can either self-associate with a monomer of the same type to produce a homodimer (IR-A/IR-A or IR-B/IR-B) or with a monomer of a different type to produce a heterodimer (IR-A/IR-B). The proreceptor dimers are then proteolytically cleaved and glycosylated, resulting in the final α and β subunits that comprise the mature IR. After synthesis, the majority of these receptors are then stored in intracellular vesicles until triggered to translocate for fusion into the plasma membrane. Both homodimers, as well as the heterodimer conformation, are capable of being successfully processed into functional IRs. However, the isoforms produced by each differ slightly in terms of their signaling properties, binding kinetics, and tissue expression, with the homodimer protein IR-B being the predominant IR in the periphery while IR-A is primarily localized to the central nervous system (CNS) [12]. As this dissertation focuses on insulin signaling in the context of peripheral metabolic dysfunction, cognitive decline and synaptic plasticity, and energy metabolism in the brain, the heterodimer isoform (IR-A/IR-B) will not be discussed here.

In spite of their differences, the IR-A and IR-B isoforms are relatively homologous in their overall structure. The mature IR is a transmembrane protein that spans across the plasma membrane and is comprised of two of α subunits and two β subunits that are linked together by disulfide bonds to form a tetrameric protein (**Figure 1.1A**). The α-subunit of the IR consists of 723 amino acids and contains two binding sites for insulin [14]. The

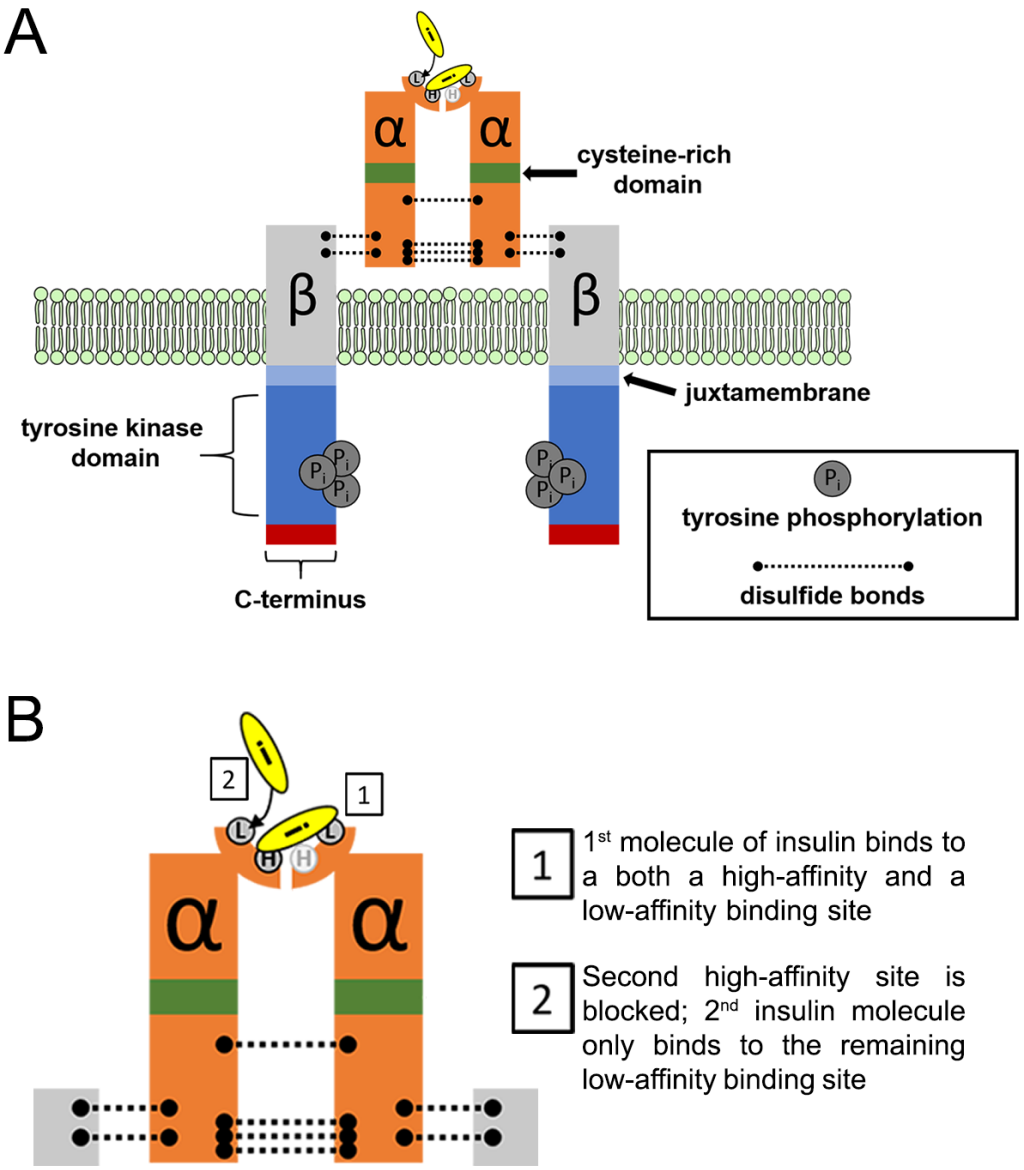


Figure 1.1. Structure of the human IR. General structure of the endogenous human IR (isoform IR-B) imbedded within a plasma membrane. **(A)** The IR is a dimer comprised of two α subunits and two β subunits held together by disulfide bonds (dotted lines). The α-subunit contains a cysteine-rich domain and resides entirely on the extracellular side of the membrane where it serves as the binding site for the ligand. The β-subunit contains a membrane-spanning domain (shown in gray), a juxtamembrane region, a tyrosine kinase

domain, and a C-terminal tail. The tyrosine kinase domain is the catalytic site of the receptor and is phosphorylated upon ligand binding. Phosphorylation of the β-subunit then triggers downstream signaling. **(B)** Close-up of the insulin binding site of the α-subunit. The IR contains both high- and low-affinity binding sites (labeled H and L, respectively). A single insulin molecule is sufficient to trigger IR signaling and will bind at the high-affinity binding site (see box 1). In situations of high ligand concentration, a second insulin molecule may then bind at the low-affinity binding site (see box 2).

catalytically active β-subunit is comprised of 620 amino acids with three distinct domains: the extracellular domain, the transmembrane domain, and the cytosolic domain [14]. The α-subunit is entirely extracellular and contains two binding sites, one high-affinity and one low-affinity (Fig. 1.1B), while the β-subunit is embedded in the lipid bilayer and protrudes into the interior of the cell where it can activate downstream signaling effectors. Catalytic activity of the IR occurs at the cytosolic domain of the β-subunit, which contains multiple tyrosine phosphorylation sites.

1.2.2 The Canonical Insulin Signaling Pathway

Transduction of the canonical insulin signaling pathway begins with the binding of insulin to the IR (**Figure 1.2**). Binding occurs relatively quickly in the periphery, with some investigators even suggesting that maximal binding is reached at ~10 min [15]. Upon binding, the IR undergoes a conformational change that induces rapid transphosphorylation of each β-subunit by the other at tyrosine residues 1158, 1162, 1163, 1328, and 1344 [16] in a process referred to as an "activation loop" [2]. Following this initial transphosphorylation event, the β-subunit also undergoes slower autophosphorylation of tyrosine residues on its upper region (juxtamembrane) and of serine residues on its intracellular C-terminus tail [2, 16]. Once active, the IR then phosphorylates tyrosine residues on a variety of intracellular targets [2, 3]. Of these, the insulin receptor substrate (IRS) family, particularly IRS-1, is likely the most well-characterized.

Following its phosphorylation by the IR, IRS-1 interacts with p85, a regulatory subunit of the enzyme phosphatidylinositol 3-kinase (PI3K), and subsequently triggers its activation and translocation to the plasma membrane [2]. Enzymatic activity of PI3K leads

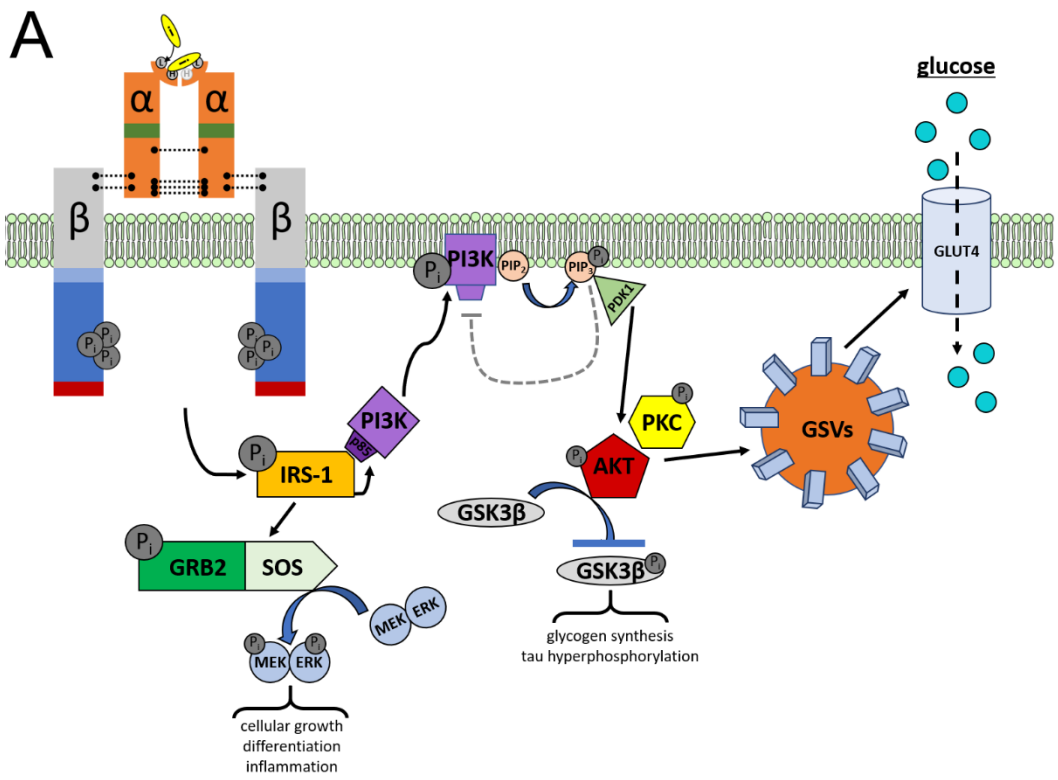


Figure 1.2. Signaling pathway of the human IR. (A) Canonical signaling pathway of the peripheral IR (isoform IR-B). Briefly, insulin binding triggers phosphorylation of the β-subunit of the IR which in turn phosphorylates IRS-1. **Right pathway:** IRS-1 interacts with the p85 subunit of PI3K, which triggers its translocation to the plasma membrane. Here, PI3K converts PIP_2 to PIP_3. PIP_3 recruits PDK1 which then activates AKT and PKC. Both AKT and PKC are involved in mediating GSV trafficking and GLUT4 translocation to the membrane. Additionally, AKT may also phosphorylate GSK3β, subsequently inhibiting it. **Left pathway:** IRS-1 may also activate GRB2 and SOS, which then phosphorylate MEK/ERK. The MEK/ERK pathway is responsible for regulating mitogenic processes such as cell growth and proliferation, differentiation, and inflammation.

to synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which can mediate the localization, trafficking, and catalytic activity of numerous intracellular proteins [17], as well as regulate the IR by inhibiting further PI3K activation [18]. PIP_3 then triggers recruitment of phosphoinositide-dependent kinase 1 (PDK1), a master kinase that regulates multiple signaling effectors including protein kinase B (AKT) and protein kinase C (PKC), both of which are involved in insulin-mediated translocation of glucose transporter (GLUT) 4 to the plasma membrane [2].

Adjacent to IRS-1/PIP_3/PI3K signaling is the mitogen-activated protein kinase 1 (MEK)/extracellular signal–regulated kinase (ERK) pathway. Phosphorylation of IRS-1 leads to recruitment of growth factor receptor-bound protein 2 (GRB2) which then associates with son-of-sevenless (SOS) and subsequently activates MEK (a member of the mitogen-activated protein kinase [MAPK] family) and ERK. Unlike the IRS-1/PIP_3/PI3K pathway, the MEK/ERK signaling cascade is primarily associated with mitogenic activity (cellular growth, differentiation and proliferation, and inflammatory processes), although some studies have found that elevated MEK/ERK activation triggered by metabolic stress can inhibit IRS-1 activation and thus attenuate IR signaling [19].

As stated in Section 1.1.2, activation and downstream signal transduction of the IR eventually triggers endocytosis-mediated internalization and processing of the ligand-receptor complex [10]. Internalization of the IR is relatively quick, with some investigators suggesting that it can occur within 30-45 min after initial receptor activation [10, 15]. Once in the endosome, the IR can either be recycled back to the plasma membrane or degraded via proteolysis; of these two potential fates, receptor recycling appears to be the most common. The exact mechanisms governing this process are still unclear, but may rely, at

least in part, on dephosphorylation of the β-subunit [10], as the receptor would need to be devoid of phosphorylated tyrosine residues in order to be successfully activated during subsequent binding events.

1.2.3 Peripheral Insulin Signaling in the Context of Obesity and Type-2 Diabetes Mellitus

Insulin signaling is an integral component of energy metabolism in the periphery due to its ability to trigger uptake of glucose into adipose and muscle tissue [2]. This is primarily regulated by a combination of AKT-mediated recruitment of GLUT4 containing vesicles (GSVs) and elevated catalytic activity of TC10, another downstream target of IR signaling that plays a crucial role in the fusion of these vesicles to the plasma membrane [20]. Briefly, elevations in blood glucose levels, as occurs following consumption of a meal, triggers release of insulin from the pancreas into the circulation. Insulin then binds to IRs on peripheral tissues which then signal through the IRS/PIP_3/PI3K pathway, leading to GLUT4 translocation and glucose uptake.

In patients with metabolic dysfunction (e.g. Type-2 diabetes mellitus [T2DM]), glucose uptake is substantially reduced due to desensitization of the IR which renders it incapable of responding to circulating insulin at a level sufficient for clearance of glucose from the bloodstream [21, 22]. This desensitization can occur in two distinct ways: 1.) the loss of functional IRs at the plasma membrane following sustained, high levels of circulating insulin (hyperinsulinemia) which triggers IR internalization/degradation [16]; and 2.) decreased responsiveness caused by receptor and/or postreceptor defects that attenuate aspects of IR signaling, such as reducing tyrosine phosphorylation of the β-subunit [16, 23, 24], decreasing activation of IRS-1 through serine/threonine

phosphorylation [24-26] and subsequent uncoupling of the substrate from PI3K/AKT [27], and increasing activation of the MEK/ERK pathway which then inhibits further IR activity [28]. Both IR internalization and decreased receptor responsiveness result in an accumulation of circulating blood glucose, otherwise known as hyperglycemia.

Hyperglycemia presents with a variety of pathological symptoms that are dependent on the severity and duration of the disease. Chronic, moderate (120-180 mg/dL) hyperglycemia occurring over many years can lead to diminished renal function, neurological impairments, damage to the extremities (diabetic neuropathy), cardiovascular dysfunction, and ketoacidosis. Acute, severe (>400 mg/dL) hyperglycemia is considered a medical emergency, and at levels of 600 mg/dL and above, diabetic coma is likely. The impact of insulin resistance on the periphery and the physiological mechanisms mediating these effects have been extensively characterized in the literature. However, it is only within the past ~30 years that hyperglycemia's effect on the CNS has been explored.

1.2.4 Impacts of Peripheral Metabolic Impairments on Cognitive Function

Peripheral insulin resistance and T2DM have recently been identified as risk-factors for cognitive impairment and dementia, particularly in older individuals [29-34]. Numerous well-powered clinical studies have indicated that elevated peripheral insulin is associated with poorer cognitive performance [35], even in patients that do not have diabetes [36]. Additionally, chronic, elevated peripheral insulin levels in combination with a prediabetic state was shown to correlate with a faster rate of cognitive decline in elderly subjects [37]. This peripheral dysfunction has also been shown to affect brain metabolism, with diabetic and prediabetic older adults having reduced cerebral glucose uptake and utilization in prefrontal, temporal, and cingulate regions compared to nondiabetic, age-

matched controls [38]. Insulin resistance has also been shown to impact brain volume [39, 40], evidenced by greater brain atrophy in T2DM patients compared to controls [41], particularly in the hippocampus and amygdala [42]. Further, it appears that the severity of brain atrophy in these patients may be positively correlated with the severity of their peripheral insulin resistance [42].

With respect to Alzheimer's disease (AD), the Rotterdam Study, the first epidemiological investigation of the relationship between T2DM and AD, reported that the risk for developing dementia was doubled in diabetic patients compared to controls [43], suggesting that sustained dysregulation of insulin-related processes in the periphery may contribute to cognitive decline and pathological changes in the brain [44]. This study is supported by work from numerous other investigators who have also provided evidence that AD is associated with peripheral hyperinsulinemia [33, 45, 46]. Investigations into the relationship between T2DM and AD bio-markers have also highlighted potential synergistic pathways linking apolipoprotein E (APOE) genotype, amyloid beta (Aβ) plaques, and neurofibrillary tangles [47, 48].

Work performed in animal models of peripheral insulin resistance has echoed this clinical data [49, 50]. An early study in genetically obese Zucker rats reported reduced levels of brain insulin compared to lean controls [51]. One report performed in C57BL/6 mice indicated that a high-fat diet (HFD) induced hepatic insulin resistance and correlated with significantly impaired synaptic plasticity [52]. Similarly, rats fed a high-fat-and-fructose diet (HFFD) for 7 days presented with markers of peripheral insulin resistance and obesity along with lower hippocampal weight, reduced dendritic arborization, and decreased dendritic spine density compared to controls [53]. These HFFD rats also had

elevated tau hyperphosphorylation, further supporting the implication that metabolic dysfunction is associated with development of AD pathology. In Wistar rats, HFD and high-sugar foods were associated with altered levels of IR signaling markers, such as PI3K and AKT, in the hippocampus and hypothalamus [54]. Additionally, our lab has shown that HFD negatively impacts markers of age-dependent calcium dysregulation in rats compared to age-matched controls [55]. Another study, this time in C57BL/6 mice fed a HFD, showed that higher peripheral insulin resistance positively correlated with markers of AD, including Aβ deposits and neurofibrillary tangles [56]. A similar report in the same animal model highlighted elevated levels of AD biomarkers such as hyperphosphorylated tau, as well as decreases in proteins associated with synaptic plasticity [57].

Use of streptozotocin (STZ) administration has also been used to measure the impact of peripheral insulin resistance on the development of AD-like pathology. Repeated administration of low-doses of STZ leads to peripheral IR desensitization and reduced IR signaling, mimicking clinical T2DM [58]. STZ animals rendered diabetic have been shown to perform poorly during hippocampal memory tasks [59-61], have reduced synaptic plasticity [59, 62, 63] and altered hippocampal gene expression [64], and show signs of elevated CNS oxidative stress, calcium dysregulation, and vascular dysfunction [59, 65].

Clearly, the current evidence strongly supports the hypothesis that peripheral insulin signaling not only mediates processes in local tissues, but also acts on pathways in the brain. However, while insulin-associated processes in peripheral tissues may be able to distantly regulate the CNS, studies within the past 30 years have suggested that the peptide can also modulate this region directly. The following section will discuss brain-specific

insulin actions, their impact on learning and memory, and the therapeutics specifically designed to target these processes in the clinic.

1.3 Insulin Actions in the CNS

1.3.1 The Brain is Insulin Sensitive

For many years, the brain was considered to be an insulin-insensitive organ; that is, one that did not possess circulating insulin, IRs, or pathways that required direct action of the ligand at the CNS. We now know this is incorrect, and that in fact, insulin and IR signaling are integral parts of normal, healthy brain function. Some of the first evidence of insulin sensitivity in the brain was derived using intracisternal insulin administration, and indicated that insulin was capable of directly acting on the CNS to regulate glucose levels in the periphery and cerebrospinal fluid (CSF) [66, 67]. Another early study reported that administration of exogenous insulin to cortical brain slices caused a small, but significant, elevation in 2-deoxyglucose uptake compared to control slices [68]. In 1978, a group of investigators provided evidence of robust, region-specific expression of IR in multiple areas of the rat brain, with the highest levels found in the olfactory bulb, cerebral cortex, anterior hypothalamus, and hippocampus [69]. Further, IR density in the brain appears to be independent of peripheral insulin levels, suggesting that insulin's actions in the CNS, while related to its peripheral counterpart in many ways, is a physiologically distinct process [70]. Work from this same lab also highlighted the presence of the insulin peptide in whole-brain preparations from male Sprague-Dawley rats; as with the IR, concentrations of the ligand were region-specific, with the highest levels detected in the olfactory bulb and hypothalamus [71]. Interestingly, the average, overall concentration of insulin in

whole-brain extracts was significantly higher (~25-fold) than the average plasma insulin level from these same animals, again implying that brain IR density and ligand levels are regulated independently from those in the periphery [71].

This early data has since been corroborated by numerous other reports of robust IR expression and insulin peptide levels in multiple areas of the brain, particularly the olfactory bulb, hypothalamus, and pyramidal cell-layer of the hippocampus [72-79]. Within these brain regions, subcellular localization of the IR appears to be largely neuronal [78], although some glial cells, such as astrocytes, may also express the receptor [80-82]. Interestingly, some data has indicated that astrocytic IRs are predominantly IR-B, the peripheral isoform of the receptor, suggesting the existence of cell-type specific IR expression and distinct signaling pathways [80, 81]. However, other labs have contradicted this finding, reporting that both neurons and astrocytes primarily express IR-A [83].

While initial studies revealed that the binding characteristics, general morphology, and kinetic profile of IRs in the brain were similar to those in the periphery [84], we now know that the brain-specific IR-A is structurally and functionally distinct from that of the peripheral isoform [12, 85]. Unlike the peripheral IR-B, IR-A does not appear to be downregulated in neurons following prolonged incubation with insulin [86]. While the β-subunit of both isoforms seem to be structurally and functionally identical, the α-subunit of IR-A is substantially smaller than IR-B (115 vs. 130 kDa, respectively) [22, 85]. IR-A also seems to have a 1.7-fold higher affinity for insulin, as well as a faster rate of ligand dissociation from the receptor [12, 13]. Although some studies have indicated that signaling my differ slightly between these two isoforms, in general, the pathways are relatively homologous, with binding of insulin triggering IRS-1 recruitment and

subsequent downstream activation of PIP_3/PI3K/AKT in both IR-A and IR-B [12]. IR signaling in the brain has been implicated as a mediator of a variety of physiological processes, including regulation of CNS energy metabolism, neuronal survival and development, modulation of synaptic plasticity and cognitive functions such as hippocampal learning and memory, and the progression of neurodegenerative disease such as AD [33, 87-93]. Of the many different pathways potentially targeted by CNS IR signaling, this dissertation will primarily focus on 1.) learning and memory processes, 2.) neuronal physiology and ion-channel activity, and 3.) hippocampal glucose metabolism, all of which will be discussed more extensively in the context of three highly relevant projects I recently completed throughout the course of my doctoral study ([94, 95]; see Chapters 2, 4, and 6).

1.3.2 Origin of Insulin in the CNS: The Role of the Blood-Brain Barrier

In order for insulin to exert direct action on the brain, it must first gain entry to the CNS. Initially, it was hypothesized that the ligand may be produced locally, as the blood-brain barrier (BBB) was believed to be impermeable to peripheral insulin [70]. This theory has since been corroborated by evidence of insulin mRNA and C-peptide in neurons [96, 97]. However, while local production of insulin in the brain may perhaps exist, the bulk of the literature strongly supports an alternative hypothesis: that the primary source of CNS insulin is transport of the ligand from the periphery across the BBB.

The suggestion that insulin could cross the BBB first arose with the observation that peripheral insulin administration was could elevate ligand levels in the CSF [98]. A later study agreed with this early research, showing that radiolabeled human insulin can cross the BBB in mice [99]. Many other groups have since confirmed these results, and

most contemporary evidence now supports the hypothesis that the majority of insulin in the brain arrives via transport of the ligand from the periphery [100]. Investigations into the kinetics of BBB insulin transport have indicated that it is a saturable system [99], and binding studies using quantitative autoradiography suggest that the majority of transport occurs at the choroid plexus and capillary beds [100, 101]. However, other work in mice reported that the fastest rate of insulin crossing at the BBB actually occurred in the olfactory bulb [73], which also coincides with this region's high density of IR expression [102, 103].

Unsurprisingly, alterations in BBB-mediated insulin transport have been correlated with metabolic dysregulation and obesity. In one study, dogs fed a HFD for 7-weeks and rendered peripherally insulin resistant had significantly decreased efficiency of insulin transport into the CNS [104]. Similarly, this same group also provided evidence of reduced transport across the BBB in dogs administered dexamethasone, a glucocorticoid known to impair IR signaling in the periphery [105]. These results are further supported by observations of reduced insulin transport following diet-induced [106] and genetically-induced [107] obesity in the Wistar and Zucker diabetic fatty (ZDF) rat models, respectively.

Interestingly, however, some studies contradict this, instead reporting increased CNS insulin transport in diabetes [108, 109] that may be caused by a loss of tight-junction integrity and elevated BBB permeability [110, 111]. Additionally, there also exists another gap in our current understanding of this process, as we have yet to determine exactly how BBB insulin transport is regulated or if it involves a specific, undiscovered insulin transporter protein [100]. While the precise cellular mechanism facilitating movement of

the ligand from periphery to brain requires further investigation, its impact on cognitive functions, particularly those mediated by the hippocampus, is indisputable.

1.3.3 Insulin and the Hippocampus: Impact on Learning and Memory

The hippocampus is a bilateral, curved formation located in the medial temporal lobe. It is part of the limbic system and is an integral component of cognitive processes such as learning and memory formation, consolidation of short and long term memory, memory acquisition and retention, and spatial navigation. Humans and rodents possess two hippocampi, one in each hemisphere of the brain. Each hippocampus can be separated into two specific regions, the dentate gyrus (DG), which is comprised of the molecular, granular, and polymorphic layers, and the hippocampus proper.

The hippocampus proper is made up of a dense network of pyramidal neurons and is separated into 4 subfields: CA1, CA2, CA3, and CA4. The CA3 subfield, which can be further divided into layers *stratum lacunosum-moleculare*, *stratum radiatium*, *stratum pyramidale*, and *stratum oriens,* is considered to be the hippocampal region most involved in learning and memory processes. The majority of signals projected into the hippocampus arise from neurons of the entorhinal cortex (EC), which enter the DG via the perforant path and synapse onto granule neurons. Granule neurons then project specialized axons, known as mossy fibers, into the hippocampus proper to form excitatory synapses at CA3 pyramidal neurons. From here, CA3 neurons project the signals down their Schaffer collaterals to CA1 neurons, which then loop back up to the EC, thus completing what is known as the trisynaptic circuit. In addition to EC neurons, the hippocampus also receives information from the medial septal nucleus, which regulates GABAergic synapses associated with hippocampal memory processes.

Impairments in hippocampal function have been detected in various disease states, such as AD and pathological brain aging, and appear to coincide with reduced hippocampal insulin signaling. As stated previously, the hippocampus is one of the primary locations of IR expression in the brain [76, 79], and markers of IR signaling have been detected in many areas of this structure, including the post-synaptic densities [112] and molecular layers of the DG and CA1 sub-field [75, 79]. Further, there is extensive evidence that IRs in the hippocampus can modulate AMPA and NMDA receptors [34, 113], improve neuronal survival [87] and synaptic plasticity [34, 93], activate key genes and signaling pathways required for long-term memory storage or short-term memory encoding [34, 114], and increase hippocampal metabolism [91, 115-118]. Two of these processes will be discussed more extensively in Chapters 3 and 5.

Behavioral tests in animal models further support the role of insulin in hippocampally-mediated learning and memory. One study reported that rats had increased IR expression and markers of insulin signaling in the hippocampus after training on the Morris water maze (MWM) behavioral test, and that STZ administration reduced these measures and worsened memory performance [119]. Metabolic dysfunction has also been associated with deficits in IR activity and hippocampal cognitive processes. A study of adult male Wistar rats fed a high-sugar diet highlighted reductions in IR signaling markers, including PI3K and AKT [54]. Additionally, 12-weeks of HFD resulted in peripheral insulin resistance as well as inactivation of IRS-1, decreased expression of GLUTs at the plasma membrane, and diminished measures of synaptic plasticity in the hippocampus of C57BL/6 mice [52]. The current data strongly suggests that insulin and IR signaling are extremely important aspects of normal cognitive function in the hippocampus. In the

following sections, I will provide a brief review of this relationship in the context of pathological brain aging and AD-associated cognitive decline.

1.3.4 Altered IR Signaling in the Context of Aging

Impaired CNS insulin signaling has been implicated as a contributor to age-related cognitive decline in both humans and animal models. Early work using ^{125}I-insulin binding assays indicated that aged rats had significantly reduced IR density in the olfactory bulb compared to young controls [120]. Another study also reported a 39% reduction in IR density in the brains of aged rats, as well as a 57% decrease in the dissociation rate of these receptors [121]. Numerous studies have since supported this initial work, providing robust evidence of reduced IR density, decreased markers of IR signaling, and lower levels of circulating brain insulin in the hippocampus, all of which have been extensively reviewed elsewhere [30, 122, 123].

While there is no denying that the aged brain exhibits reduced IR activity, whether this is caused by an inability of the receptor to activate effector proteins downstream of its signaling pathway or if it instead stems from a lack of circulating insulin available for binding remains a point of contention [122]. Many investigators believe that the aged brain experiences a type of insulin resistance similar to that experienced in the periphery, particularly in individuals who have comorbid metabolic dysfunction. Yet some evidence does exist of preserved insulin sensitivity in these phenotypes. A study in the ZDF rat indicated that diabetic rats had normal memory function during a behavioral test and preserved synaptic plasticity [124]. While this particular study did not specifically look at aged animals, it does provide evidence that peripheral insulin resistance does not necessarily correlate with brain insulin resistance. More recent work, this time utilizing

young and aged APP/PS1 mice, showed that insulin could still elicit normal signaling activity, provided it was delivered directly to the brain [125]. Prior work from our own lab reported that hippocampal brain slices from aged rats rapidly responded when administered exogenous insulin, and in one case, actually responded more robustly on measures of calcium-dependent processes compared to slices from younger animals [55, 126]. These results were further supported by a later study in our lab that showed aged animals receiving acute insulin administration to the brain had significantly higher increases in cerebral blood flow (CBF) compared to young [127].

A possible explanation for this phenomenon could be that aged animals simply have reduced transport of insulin across the BBB, thus decreasing IR signaling without impacting receptor sensitivity. This hypothesis is supported by recent evidence of aged mice having no changes in brain IR signaling following a peripheral subcutaneous injection of insulin, but responding robustly once the ligand was administered directly to the brain [128]. While the debate continues on the subject of age-related insulin insensitivity in the brain, the bulk of the literature still supports the hypothesis that IR signaling is reduced by aging and metabolic dysfunction. However, these are not the only pathologies that exhibit this phenomenon. Considerable evidence of impaired insulin activity has also been discovered in the brains of AD patients, leading to the development of a theory known as The Type-3 Diabetes Hypothesis.

1.3.5 Brain Insulin Resistance and AD: The Type-3 Diabetes Hypothesis

The first substantial data suggesting an association between brain insulin resistance and AD pathology was discovered in post-mortem tissue. This study showed that brains of AD patients had significantly lower levels of IR signaling and insulin concentrations

compared to age-matched, healthy controls [129]. Paradoxically, these investigators also reported that AD patients had elevated IR expression; this has since been hypothesized to be a compensatory mechanism to counteract the reduction in circulating insulin. These early results were in agreement with work from Craft and colleagues, which stated that AD patients had elevated plasma insulin and lower CSF insulin than healthy controls, and that these differences were positively correlated with AD severity and APOE genotype [130]. These observations lead to the formulation of the brain insulin resistance hypothesis of AD, which suggests that CNS insulin resistance contributes to the development of AD pathology [131]. In 2003, de la Monte and colleagues proposed the term "Type-3 diabetes" to reflect the similarity of this process to the development of T2DM in the periphery [132]. Numerous other contemporary studies have since support this hypothesis, citing evidence of aberrant insulin signaling, elevated markers of impaired IR responsiveness, lower IR density, and reduced concentration of the ligand in AD patients [49, 122, 133-138]. However, the exact mechanism by which reduced insulin activity affects the pathogenesis of this disease remains unclear.

A substantial amount of work has proposed that impaired insulin signaling may contribute to AD development through its ability to regulate biomarkers of disease pathology. Recent studies have provided evidence of this link in neuronal cultures, highlighting that direct administration of insulin reduced the amount of Aβ accumulation these cells [133, 138]. IDE, which is mediated by IR activation and subsequent phosphorylation of PI3K [34], may also play a role in this process, as it has been shown to degrade Aβ [133, 135, 138]. Aβ oligomers have also been reported to reduce the number

of hippocampal IRs and worsen brain insulin resistance, further supporting the association between insulin-related processes and AD development [34].

IR signaling has also been linked to the regulation of neurofibrillary tangles. These tangles are formed by hyperphosphorylation of tau, which causes aggregation of the protein in axons and synapses of neurons [133, 138]. It has been proposed that this excess phosphorylation is triggered by an overactivation of glycogen synthase kinase-3 beta (GSK-3β). Binding of insulin to the IR and downstream activation of AKT inhibits GSK-3β. Therefore, the reduction of IR signaling in the AD brain [134] may lead to elevated GSK-3β activity, thus increasing the level of tau hyperphosphorylation and associated neurofibrillary tangles [133]. This theory has been supported by work in animal models showing that STZ- or HFD-induced brain insulin resistance significantly decreases hippocampal IR signaling, downregulates synaptic and dendritic proteins, and significantly elevates both total and hyperphosphorylated levels of the tau protein [57, 139].

Impaired insulin signaling may also contribute to AD development by reducing CBF. AD patients have significantly decreased regional CBF compared to age-matched controls [133]. IR signaling has been shown to mediate CBF through activation of the PI3K/AKT pathway, which then triggers vasoconstriction or vasodilation in the CNS [133]. Further, reduced CBF can result in elevated oxidative stress and inflammation, both of which also contribute to neurodegeneration and cognitive decline associated with AD development [133].

To combat these pathogenic processes, the use of exogenous insulin administration, either delivered peripherally or directly to the brain and CNS, has recently been explored as a clinical therapeutic. Studies performed in both human patients and animal models have

been optimistic, reporting improvements in cognitive function, elevated activation of insulin-related processes, and decreases in markers of pathogenic brain aging or AD after treatment [140, 141]. In this next section, I will present a summary of several important studies regarding this therapy and provide a brief overview of commonly used insulin-delivery techniques.

1.4 Targeting Alzheimer's Disease and Age-Related Cognitive Decline with Exogenous Insulin

1.4.1 Periphery to Brain

Evidence of reduced CNS insulin signaling and decreased insulin transport across the BBB in patients with T2DM and/or AD initially investigators to try to target brain IRs by raising peripheral insulin levels. An early study in men receiving intravenous (IV) infusion of insulin provided the first evidence that CSF insulin levels were elevated following peripheral treatment with the ligand [142]. In the clinic, Craft and colleagues showed that peripherally-induced hyperinsulinemia using IV insulin infusion in AD patients could improve declarative memory and selective attention compared to age-matched controls, even in the absence of hyperglycemia [143, 144]. These same investigators also showed that elevated plasma insulin improved memory performance in both cognitively normal adults and in AD patients, and that the level of enhancement was dependent on dose, disease severity, and APOE genotype [145]. Another study from a different group reported that IV infusion of "high" levels of insulin (15 mU/kg per min) was associated with significant improvements on the word recall and Stroop selective attention tests compared to "low insulin" (1.5 mU/kg per min) [146]. These results were

further corroborated by evidence of memory improvement in adults following IV infusion of the ligand [147]. Interestingly, however, this study also reported that IV insulin increased the amount of CSF Aβ, and that improvements in memory performance were only detected in younger individuals who did not exhibit high levels of this protein, suggesting that physiological changes associated with AD pathology may alter the efficacy of insulin on learning and memory processes.

In the STZ animal model of T2DM, preemptive subcutaneous insulin injection lead to an attenuation of STZ-associated behavioral deficits but was not able to restore behavioral performance if given after these deficits had already developed [60]. A later study in STZ rats also using subcutaneous insulin infusion showed that important components of memory and synaptic plasticity, such as neurotransmitter activity and synaptic potentials, were protected from STZ-associated impairments [148]. Another group, this time utilizing the ZDF rat model, provided evidence of a potential protective function of hyperinsulinemia, showing that ZDF animals had normal learning and memory on the MWM and preserved markers of hippocampal synaptic plasticity [124]. This work is supported by a similar study which reported no signs of neuroaxonal dystrophy, a common symptom of T2DM, in this same animal model [149]. Conversely, early work in obese ZFD rats highlighted a substantial decrease in peripheral to CSF insulin transport following hyperinsulinemia rather than an increase [107], and many others have reported a detrimental effect of elevated peripheral insulin levels on brain IR signaling and cognitive function [36, 125, 150], suggesting there may be multiple factors influencing the actions of peripheral insulin on the CNS. Regardless, these studies strongly imply that targeting the brain with exogenous insulin administration is a promising strategy to improve memory

and learning while reducing cognitive decline associated with brain insulin resistance. However, as IV or subcutaneous ligand administration requires transport across the BBB in order to exert actions on the CNS and is confounded by the detrimental symptoms it can cause in the periphery (e.g. hypoglycemia), other, more direct, techniques have since been explored.

1.4.2 ICV Insulin Administration

The method of intracerebroventricular (ICV) drug delivery allows investigators to bypass the BBB by injecting the ligand of interest directly into the CSF where it can then travel unimpeded to the brain. An early study of ICV insulin in Sprague-Dawley rats showed no changes in plasma insulin levels, body weight, or food intake, suggesting that it is a relatively safe method for increasing CNS insulin levels without affecting peripheral metabolism [151]. Another study in this same model showed that insulin delivery using ICV resulted in elevated hippocampal IR signaling and stimulated GLUT4 translocation in a PI3K-dependent manner [152]. Further, ICV insulin significantly improved memory retention on a step-through passive avoidance task compared to saline controls in male Long-Evans rats [153].

A study performed in the Fisher 344 (F344) animal model of aging reported that memory impairments and neuroinflammation associated with lipopolysaccharide (LPS) were attenuated by ICV insulin in younger, but not older, rats, highlighting the significance of age on insulin's CNS effects [154]. More recent work echoed these results, with ICV insulin reversing LPS-induced elevations in inflammatory markers, oxidative stress, and cognitive impairments in male Wister rats [155]. Another study in Wistar rats showed that

ICV insulin could significantly improve spatial learning and memory compared to saline controls, but only at higher doses (16 and 32 mU) [156].

While insulin's beneficial impact on cognitive function is strongly supported, the use of ICV to administer the ligand is not a feasible therapeutic for most clinical situations. Due to this, many investigators recognized that a safe, effective, and relatively noninvasive method for introducing insulin directly into the brain was needed. Thus, a new protocol for targeted drug delivery was developed.

1.4.3 Intranasal Insulin: A Superior Technique for Direct Delivery of Ligands into the Brain

The use of intranasal drug delivery to bypass the BBB was first detailed by Frey and colleagues using wheat-germ agglutinin-horseradish peroxidase (WGA-HRP) in Sprague-Dawley rats. This initial study reported that WGA-HRP was highly concentrated in the olfactory bulb following administration, providing the first quantitative evidence of intraneuronal transport using the olfactory route [157]. In 2001, this same group showed successful transport of insulin-like growth factor 1 (IGF-1) into the brains of Sprague-Dawley rats following intranasal delivery, also noting a positive impact of the drug on cognitive deficits associated with stroke [158].

Three routes for entry of intranasally delivered peptides into the brain have been proposed: 1.) the intraneuronal route, in which the drug is directly internalized by the olfactory neurons, trafficked to the first order synapses of the olfactory bulb via axonal transport, and exocytosed for transsynaptic transfer to other cells along the route [157]; 2.) the extraneuronal route, in which the drug passes through intercellular clefts between tight junctions of the olfactory epithelium and subsequently diffuses into the subarachnoid

space [159]; and 3.) the trigeminal route, in which the drug migrates across the lamina propria through openings in the cribriform plate and enters the perivascular/perineural space of the trigeminal nerve, where it then travels to the CSF [160]. While evidence supporting the intraneuronal route for many peptides has been reported, this process would require a substantial amount of time (~24 h) to deliver the drug into the brain [159] and would also significantly increase the possibility of proteolysis of the peptide within the intracellular space [161]. Studies have indicated that intranasal administration of larger molecular weight drugs results in a relatively rapid elevation of drug concentrations in the CNS [161]; therefore, it is unlikely that the intraneuronal route is the primary route for peptides of this size to enter the CNS. With respect to insulin specifically, most evidence now supports the trigeminal route of intranasal delivery [162].

The relative ease of administering peptides intranasally has made it an attractive method for clinical therapies. However, the technique understandably raises concerns that the drugs may still manage to enter the bloodstream and cause dangerous peripheral effects. Fortunately, numerous studies have shown that upon administration to nasal mucosa, the peptides immediately bypass the BBB and do not subsequently re-enter the bloodstream or cause any notable deleterious effects on peripheral processes. This evidence has been reported in both animal models, as well as in the clinic, particularly with regard to intranasal insulin (INI), which has repeatedly shown no impact on blood glucose or plasma insulin levels in human subjects following delivery of the ligand [126, 127, 163-165]. Since these original studies, numerous investigators have explored the efficacy of INI as a reliable, rapid, and selective method for targeting insulin resistance and related pathogenic processes in aging and AD.

1.4.3.1 INI in the Clinic

Over the past 20 years, numerous studies of INI in the clinic have highlighted a beneficial impact on peripheral metabolism and CNS process [163, 166-169]; however, it wasn't until 2004 that the first substantial investigation of this therapy's impact on cognitive function was reported. This study was the first to show that INI could significantly improve memory and learning [170]. Later work in patients with AD (early onset and amnestic MCI) echoed these results and highlighted the importance of APOE genotype on the efficacy of treatment, reporting that while INI improved verbal memory performance in both APOE-ε4+ and APOE-ε4- individuals as well as cognitively healthy adults, the beneficial effect was much greater in patients with the ε4- allele [164]. Additionally, ε4+ patients actually performed worse on a third memory test after treatment, suggesting that AD pathology plays a substantial role in how the brain responds to this particular therapy. Later results from a clinical pilot trial performed in younger patients with amnestic MCI and older patients with mild to moderate AD indicated that both 20 and 40 IU of INI preserved general cognition (ADAS-cog scores) in the younger amnestic group and improved functional abilities (ADCS-ADL score) in older AD patients [171]. Both groups had enhanced performance on the delayed memory test, albeit only at a dose of 20 IU. Additionally, CSF analysis revealed that memory and function improvements were associated with changes in overall Aβ-42 levels and Aβ-42/tau ratios in these patients. INI also substantially improved glucose uptake, hinting at a potential mechanism of action of this therapy [171]. Similar results regarding INI's impact on learning and memory in AD or MCI patients have since been reported by multiple investigators [172, 173], who

have also presented evidence of the potential modulatory impact of sex on INI's efficacy [174, 175] as well as INI's ability to improve cerebral blood flow (CBF) [176].

While clinical trials have primarily focused INI as a treatment for AD and related pathologies, the beneficial impact of this therapy is not limited to only these patients. An early study in cognitively healthy adults showed that INI of human regular insulin (40 IU, 4 times a day for 8 weeks) had a small, but positive, impact on delayed word list recall, significantly boosted mood, and improved self-confidence compared to placebo controls [170]. Importantly, treatment with 40 IU of INI did not appear to alter components of peripheral metabolism, such as blood glucose or plasma insulin levels, bolstering previous evidence of INI's safety in the clinic [161]. Another report, also performed in healthy adults using 40 IU of INI, indicated that insulin administration could improve delayed odor-cued reactivation of spatial memory and again highlighted no changes in blood glucose or insulin levels following treatment [177]. In study of peripheral metabolic dysregulation, INI enhanced vasoreactivity, visuospatial memory and verbal fluency in both healthy controls and patients with T2DM [178]. Similar to a previous study in AD patients [176], INI was also shown to increase CBF in younger, as well as older, cognitively healthy adults [179].

Studies of INI in the clinic are currently ongoing, and the bulk of the literature strongly supports this therapy as being a reliable, rapid, and effective method for insulin administration in the brain. These results have been extensively reviewed [31, 141, 180-186], yet the exact mechanisms involved in INI's modulation of cognitive processes such as learning and memory cannot be thoroughly explored using only clinical trials and post-mortem tissue. For this reason, many investigators have turned to animal models to better elucidate these pathways.

1.4.3.2 INI in Animal Models

Work performed in AD animal models has yielded results that largely mirror those from the clinic. Seven days of consecutive INI administration in 3xTg-AD mice was shown to restore markers of insulin signaling (pIRβ, PI3K, and pAKT), elevate the expression of synaptic proteins (synapsin 1, PSD95, and synaptophysin), inhibited activation of astrogliosis, and reduced levels of Aβ-40 in the forebrain [187]. A similar study reported that INI enhanced cognitive performance on the MWM, improved markers of brain IR activation, and reduced oxidative stress, tau hyperphosphorylation and Aβ accumulation in the hippocampus and cortex of both adult and aged 3xTg-AD mice [188]. Banks and colleagues reported that in the SAMP8 mouse model of AD, INI significantly improved memory (both acquisition and retention) on the T-maze cognition assessment and object recognition compared to vehicle controls [189]. Another study in this same model reported that long-term INI (daily administration for 37 or 56 days) using recombinant human insulin slowed early-stage progression of AD-like memory loss but was not able to improve performance after greater levels of Aβ accumulation and cognitive dysfunction had occurred [190]. Work performed in 6-month old APP/PS1 AD mice indicated a positive impact of INI on anxiety-like behavior and spatial memory plasticity, as well an INI-associated amelioration of aberrant brain insulin signaling, reductions of Aβ plaques and elevations in Aβ degradation, and enhanced neurogenesis [191]. Further, a study performed in a model of sporadic AD (low-dose STZ administration) showed that INI restored cerebral glucose metabolism, attenuated astroglia activation, and reduced neuronal loss [116].

With regard to other animal models, INI was shown to improve cognitive performance on the radial arm water maze and increase PKCγII expression in the hippocampus of wild-type C57BL/6 mice [87]. Interestingly, another study of wild-type CB57BL/6 mice reported that chronic, long-term (30 or 60 days) INI did not improve odor-discrimination or olfactory learning, suggesting that the duration of treatment may impact its efficacy [192]. Anesthesia-induced impairments in spatial learning and memory performance on the MWM were also reduced following INI in the C57BL/6 mouse model, as were markers of insulin resistance, impaired synaptic plasticity, and hyperphosphorylated tau levels [193]. Following traumatic brain injury, INI significantly improved MWM performance and brain glucose metabolism while also attenuating hippocampal lesion volume and glial activation in Sprague-Dawley rats [194]. INI was also shown to improve anxiety-like behavior and reduce glial activation and neuroinflammation in the hippocampus of methamphetamine-treated rats [195]. In a feline animal model, a study of HIV-associated neurodegeneration using FIV-infected cats reported that INI was able to reduce markers of inflammation and glial cell activation, preserve cortical neurons, and enhance behavioral performance [196].

In rats rendered diabetic (STZ-induced T2DM), INI administration ameliorated diabetes-associated spatial memory deficits on the MWM task, resulting in a 3-fold decrease in latency in INI-treated rats compared to untreated diabetic animals [197]. Similarly, diabetic Sprague-Dawley rats receiving INI had improved performance on the MWM, elevations in IR signaling markers (pIRS-1, pAKT, pGSK3β), decreased glial cell activation, neuroinflammation, and Aβ-42 expression, and prevention of postsynaptic neurotoxicity [198]. This group also showed that INI could improve CBF, reduced

oxidative stress, and improve mitochondrial function in this same model [199]. Work from another group, also performed in STZ-induced diabetic rats, reported that INI using human recombinant insulin (5 IU/day for 6 days) significantly reduced levels of CSF Aβ in both diabetic and control animals [200], mimicking clinical results that highlighted INI's ability to improve memory in healthy adults [170, 177]. In a STZ-induced Type-1 diabetes model, both 5-month old control and 5-month old treated (0.48 IU of INI per day for 15 weeks) male Wister rats had significantly improved spatial learning on the MWM [201]. Interestingly, 1.5-month old rats in this study, either diabetic or control, did not appear to respond to INI treatment, suggesting that age may influence INI's efficacy. Another study in Wistar rats, this time rendered diabetic through a combination of diet and STZ treatment, showed that 4-weeks of INI significantly reduced the level of hyperphosphorylated tau in diabetic animals and completely reversed diabetes-associated markers of brain insulin resistance, particularly in the hippocampus [202].

With respect to aging, our lab has performed a series of INI studies in the F344 rat model. In one, we highlighted the beneficial impact of two formulations of insulin: lispro and detemir. Following a low-dose INI treatment with either formulation, aged F344 animals were indistinguishable from their younger counterparts on measures of MWM spatial learning and memory [126]. However, in similar study, this time using insulin glulisine, neither acute nor chronic INI treatment significantly improved memory or recall, although it did increase IR signaling and improve CBF in the aged animals [127]. While disappointing, these results align with prior work in wild-type mice that also showed no improvements in memory following long-term, chronic INI [192], again supporting the notion that treatment duration is an important component of INI's efficacy.

The results from our initial studies in the F344 animals, though contradictory, were still encouraging, as it was clear that insulin was able to successfully bypass the BBB, elevate IR signaling in the aged brain, and, at least in some cases, improve cognitive function and CBF. Additionally, other work from our lab using *ex vivo* administration of insulin to hippocampal slices from young and aged animals, as well as *in vitro* delivery to primary hippocampal cultures, has also provided promising results that highlight insulin's impact on calcium-dependent processes and glucose metabolism [55, 126, 203, 204]. Because of this, we chose to continue our work in the F344 animal model of aging and explore INI's effect on cognitive function using another insulin analog: aspart. The following chapter presents a study on the impact of this formulation on aspects of hippocampal-mediated spatial learning and memory using the MWM, as well as its ability to modulate hippocampal IR expression and alter the gene transcriptome.

The following manuscript has been published in the Journal of Gerontology: Series A: J Gerontol A Biol Sci Med Sci. (2019). Epub 10 June 2019. doi.org/10.1093/gerona/glz105. I helped perform all INI administrations, animal behavioral assessments, perfusions, and whole-brain extractions. Authors K. L. Anderson and A. O. Ghoweri assisted with intranasal delivery, behavioral testing, and data analysis; E. Sudkamp and J. R. Pauly performed ^{125}I-insulin receptor autoradiography and data analysis; A. O. Ghoweri, E. S. Johnson, K. E. Hargis-Staggs, and E. M. Blalock performed RNA extraction and microarray data analysis. Authors K. L. Anderson, G. A. Fox, K. Vatthanaphone, M. Xia, and R.-L. Lin performed immunohistochemistry experiments and data analysis. In this study, I sought to explore the following hypotheses: 1) that insulin aspart, a clinically-relevant insulin formulation that has shown enhanced brain penetration, would ameliorate cognitive decline in aged F344 rats; 2) that long-term, repeated, daily INI across 3 consecutive months would alter IR expression and hippocampal function; and 3.) that downregulation of IRs in the hippocampus or elsewhere following chronic INI is more pronounced in aged animals compared to young. While prior studies have tested the effects of INI aspart and/or other insulin formulations on spatial memory and IR expression in animal models [126, 127, 187, 193, 197, 201], the following work details novel and clinically-relevant findings regarding the effect of long-term insulin administration on hippocampal function, and provides the first comprehensive analysis of the hippocampal transcriptome in aging that is sensitive to INI aspart.

CHAPTER 2. LONG-TERM INTRANASAL INSULIN ASPART: A PROFILE OF GENE EXPRESSION, MEMORY, AND INSULIN RECEPTORS IN AGED FISHER 344 RATS

H. N. Frazier[1], A. O. Ghoweri[1], E. Sudkamp[2], E. S. Johnson[1], K. L. Anderson[1], G. A. Fox[1], K. Vatthanaphone[1], M. Xia[1], R.-L. Lin[1], K. E. Hargis-Staggs[1], N. M. Porter[1], J. R. Pauly[2], E. M. Blalock[1], and O. Thibault[1]

[1]Department of Pharmacology and Nutritional Sciences, University of Kentucky College of Medicine, 800 Rose St., Lexington KY 40536, USA

[2]College of Pharmacy, University of Kentucky, 789 S. Limestone, Lexington, KY 40536, USA

2.1 ABSTRACT

Intranasal insulin is a safe and effective method for ameliorating memory deficits associated with pathological brain aging. However, the impact of different formulations and the duration of treatment on insulin's efficacy and the cellular processes targeted by the treatment remain unclear. Here, we tested whether intranasal insulin aspart, a short-acting insulin formulation, could alleviate memory decline associated with aging and whether long-term treatment affected regulation of insulin receptors and other potential targets. Outcome variables included measures of spatial learning and memory, autoradiography and immunohistochemistry of the insulin receptor, and hippocampal microarray analyses. Aged Fisher 344 rats receiving long-term (3 months) intranasal insulin displayed a trend towards improved recall on the Morris water maze task. Autoradiography results showed that long-term treatment reduced insulin binding in the thalamus but not the hippocampus. Results from hippocampal immunofluorescence revealed age-related decreases in insulin immunoreactivity that were partially offset by intranasal administration. Microarray analyses highlighted numerous insulin-sensitive genes, suggesting insulin aspart was able to enter the brain and alter hippocampal RNA expression patterns including those associated with tumor suppression. Our work provides insights into potential mechanisms of intranasal insulin and insulin resistance, and highlights the importance of treatment duration and the brain regions targeted.

2.2 INTRODUCTION

Intranasal insulin (INI) has become a well-recognized method for addressing numerous neurodegenerative conditions. Several labs have provided evidence that it is a favorable and relatively noninvasive technique for selective delivery to the brain [157, 161, 164, 184, 185, 194, 205-207]. Using methods developed by Frey and colleagues (Frey WH II, Method for administering insulin to the brain; Patent #6,313,093 B1, issued November 6th, 2001), INI's potential as a therapeutic for mild cognitive impairment (MCI)- or Alzheimer's disease (AD)-associated memory decline has been investigated in both clinical and pre-clinical studies. Although differences in responses based on sex [167, 168, 174, 175] and APOE genotype [164, 172, 173, 175] have been noted, clinical trials have been encouraging, citing both the safety of INI, as well as its positive impact on memory function [170, 177] and components of peripheral metabolism [208, 209]. Early work from Craft and colleagues highlighted INI's impact on memory, reporting that individuals with early AD or amnestic MCI receiving 20 international units (IU) of INI for 21 days retained more verbal information than controls [210]. Recently, 21 days of INI detemir (40 IU), a long-lasting insulin analogue, improved memory performance [172]. However, the cognitive benefits of INI are not limited to only AD or MCI patients. In a study of 38 healthy male subjects (aged 18-34 years), 8-week INI administration correlated with improved immediate and delayed word recall, attention, and mood [170]. This was corroborated by improved word-list recall in healthy male subjects following INI using the rapid-acting insulin analogue aspart [207]. Together, these studies highlight the important role of insulin in both declarative and spatial memory and suggest INI may target areas associated with these processes. Potential mechanisms suggested to underlie these actions

include alterations in glucose metabolism [116, 194, 211], reductions in inflammation and glial cell activation [154, 187, 194, 198], and a rapid and reliable increase of cerebral blood flow (CBF) [176, 178, 179, 212]. This latter mechanism is particularly interesting as healthy young subjects also respond favorably to INI. However, the nature of clinical studies limits their ability to identify mechanistic processes [213]; thus, further analyses in animal models are required.

Following clinical studies, investigations of INI in animals have used models mimicking early-stage AD or MCI [214]. Critical work from Banks and colleagues reported transport of INI into the brain parenchyma, while relatively low [189], is nevertheless effective and long-lasting, as no efflux mechanisms for the peptide appear to exist [73, 215]. These same investigators recently reported that ligand transport did not differ between AD-like (older SAMP8) or AD-predisposed (younger SAMP8) mice compared to healthy controls [216], suggesting INI is a viable method for elevating insulin in the brain regardless of AD status or severity. Other work from the same group highlighted improvements in task acquisition in 12-month old male SAMP8 mice exposed to INI 24 h prior to T-maze testing, as well as 5 min or 24 h after training on a 7-day retention task [189]. Additionally, INI appears to slow the progression of memory loss in earlier stages of AD in younger (< 6-month old), but not older (> 6-month old), male SAMP8 mice [190]. In a study using the 3xTg mouse model, INI improved spatial memory recall and increased insulin signaling [188]. Another group showed that in female 3xTg mice, INI reduced amyloid beta levels and microglial activation [187], and improved cognitive performance, neurogenesis, and insulin signaling in female APPswe/PS1dE9 mice [191]. With respect to aging, Apostolatos and colleagues have shown improved

spatial memory on the radial arm water maze following 4 weeks of daily INI with human recombinant insulin in aged (18-month old) male C57Bl6 mice [87]. Studies of older (16-18-month old) female mice showed that 7-day INI prevented anesthesia-induced reductions in spatial memory performance while reducing tau hyperphosphorylation [193]. Fadool and colleagues have provided evidence that short-term (5-day) INI human recombinant insulin increases novel object recognition and odor discrimination in 2-month old male C57Bl/6 mice [217]. However, this effect was not present when extended exposures (30-60 days) were used in 5-month old animals [192], suggesting that duration may impact efficacy. We have investigated the ability of INI lispro and detemir to offset age-related cognitive decline in the Fischer 344 (F344) rat model of aging. In these studies, INI improved memory recall of the platform location on the Morris water maze (MWM) test in aged animals [126]. Another study in the same animals using INI glulisine did not improve performance, but did facilitate mechanisms that could promote memory, including increasing CBF and insulin receptor (IR) signaling in aged animals [127]. Overall, the evidence appears to robustly support the hypothesis that insulin is involved in memory processes in both humans and animal models, and makes a strong case for INI as a clinically-relevant therapy to ameliorate age- and/or AD-associated cognitive decline. However, the impact of insulin formulation and treatment duration on the efficacy of INI, as well as the cellular mechanisms targeted by this approach, remain unclear.

The premise of our work has been to address age- and AD-related reductions in insulin activity, represented by declining insulin concentrations and IR density [120, 129, 218], with a restorative increase in brain insulin levels using INI. As recently highlighted, the elements responsible for brain insulin insensitivity include diminished IR signaling

through its canonical pathway (i.e. IRS1, GSK3β), a reduction of the ligand in the brain, and/or decreased insulin transport at the blood-brain barrier (BBB) [reviewed in 122]. Since these mechanisms are likely connected, alterations in any of these processes could lead to reduced insulin signaling or function. Here, we present a series of experiments designed to test: 1) whether insulin aspart, a clinically-relevant formulation that has shown enhanced brain penetration [207, 219], could alleviate cognitive decline in F344 animals, and 2) if repeated, daily INI across 3 consecutive months could cause changes in IR expression and modify insulin's impact on hippocampal function. We also investigated the hypothesis that downregulation of IRs in the hippocampus or elsewhere following chronic INI is more pronounced in aged animals. The following outcome variables were obtained: spatial learning and memory and reversal learning, IR autoradiography and immunohistochemistry (IHC), and hippocampal microarray analysis. Results show a trend toward improved performance on the MWM reversal probe test in animals treated with INI aspart. The treatment significantly reduced ^{125}I-insulin binding in the thalamus, but not in the hippocampus. Hippocampal immunofluorescence revealed a significant age-related decrease in IRs in *stratum pyramidale* and *oriens* with a trend of INI-mediated increases in *stratum pyramidale*. Hippocampal microarray analyses identified several pathways sensitive to INI, including novel genes associated with tumor suppression, neurogenesis, and synaptic stabilization.

2.3 Materials and Methods

2.3.1 Animal Models

The work strictly adhered to the regulations of our institutional licensing committee for the care and use of animals (Institutional Animal Care and Use Committee). 22 young (2-month old) and 26 aged (18-month old) male F344 rats were obtained from the National Institute on Aging colony. One young and one aged animal died within a week of arrival. Animals were housed in pairs except for the two animals that lost their cage mates. Animals were tail marked for identification, maintained on a 12 h ON, 12 h OFF light schedule, and fed Teklad global 18% protein rodent diet *ad libitum* (2018; Harlan Laboratories, Madison, WI). As expected, young animals gained weight during the duration of the study. Aged animals neither gained nor lost weight. INI aspart did not affect animal weights (data not shown). Experimenters were blinded to the treatment groups and codes were only revealed after statistical analyses were performed.

2.3.2 Intranasal Insulin Delivery

Intranasal delivery of insulin aspart to the remaining 46 animals began 1 week after arrival and followed our previously published protocol [126, 127]. Animals were transiently held supine in a DecapiCone (Braintree Scientific, Braintree, MA) while two 5 µL doses of either sterile saline or insulin aspart (NovoLog®), given 1 min apart, were delivered to the right naris using a P10 pipette. INI continued for 3 months (62-64 doses per animal: 5 days a week, once a day, for 12 weeks). Insulin aspart was made fresh weekly and diluted from a U-100 vial (Novo Nordisk Inc, Plainsboro, NJ) using sterile saline. We

chose a concentration of 0.0715 IU/10 μL as it mimics the approximate dosage used in numerous clinical trials.

2.3.3 Spatial Behavior

On the 12th week of INI, animals (aged 5 or 21 months) underwent a spatial learning and memory test using the MWM. The testing pool measured 190 cm in diameter. A 15 cm escape platform was placed 1.5 cm below the water's surface. Water was maintained between 25 and 26 °C and made opaque to hide the platform using black tempura paint. A semi-random drop location was used for each trial. Animals were allowed 60 s to find the platform, after which they were guided to its location by the investigator. A Videomex-V acquisition and Water Maze analysis software (v4.64, Columbus Instruments, Columbus, OH) was used to track and measure movement. Each animal remained on the platform for 30 s before returning to a heated holding chamber for ~2 min.

On the first day of MWM, a visual acuity test was performed with a white cup placed above the partially submerged platform for 3 consecutive trials. Three animals in the aged insulin aspart group failed to find the platform in all trials and were removed from the behavioral analysis. After a 2-day rest, animals were subjected to 3 training trials per day for 3 days (~2.5 min intertrial interval). Twenty-four h after the last training day, a probe trial was initiated with the platform removed (up to 60 s of max swim time). The following day, the platform was place in the opposite quadrant and animals were trained on its new location (reversal learning). After 72 h, a reversal probe trial was initiated with the platform removed. Following behavioral testing, brains from 20 animals were used for autoradiography measures. Brains from the remaining 26 animals were hemisected; left

tissues (hemispheres) were used for IR IHC and right tissues (whole hippocampi) were used for microarray analyses.

We present behavioral data on path length measures and number of platform crossings on the 24-h memory recall (probe), as well as time in goal quadrant in the first 30 s of the 72-h reversal probe, from 43 animals (young saline n = 11, young aspart n = 10, aged saline n = 13, aged aspart n = 9). Swim speed was averaged from the 3 trials on the 3rd training day. As previously presented [126, 127] aged animals swam more slowly than young ($F_{(1,39)} = 24.9$, $p < 0.0001$; data not shown) and INI did not have an impact on swim speed ($F_{(1,39)} = 0.4$; $p > 0.05$).

2.3.4 ^{125}I-Insulin Receptor Autoradiography

Whole brains, including olfactory bulbs, were extracted from randomly selected animals (n = 5 per group) following anesthesia (5% isoflurane). Brains were placed on finely crushed dry ice, covered by the ice, and submerged in chilled 2-methylbutane. Tissues (16 µm sections) were mounted on slides and prepared for ^{125}I-insulin receptor autoradiography using the assay described by Kar and colleagues [76]. Briefly, slides were incubated for 18 h in 10 mM HEPES buffer (pH 8.0) containing 0.5% BSA, 0.025% bacitracin, 0.0125% N-ethylmaleidimide and 100 kIU aprotinin (Sigma Aldrich, Saint Louis, MO), and 25 pM ^{125}I-insulin (2000 Ci/mmol; PerkinElmer, Waltham, MA) at 4 °C, then washed twice (5 min each, 4 °C) with 10 mM HEPES buffer (pH 8.0). Slides were then washed once with a 10-fold dilution wash buffer and again with deionized water (10 s each, 4 °C), air dried, and stored in a vacuum-sealed desiccator. The next day, slides were incubated on tritium-sensitive film (Amersham™ Hyperfilm™ MP; GE Healthcare, Chicago, IL) and stored in X-ray cassettes for 2 months. Films were then processed in a

Kodak D-19 Developer for 5 min, run through a 30 s indicator stop bath, and exposed to Kodak rapid fixer for 5 min. Images were captured using a Northern Lights desktop illuminator (Model B95; Imaging Research, Ontario, Canada) and a Sony XC-77 CCD camera via Scion LG-3 frame grabber. ImageJ v1.59 (National Institutes of Health) was used for quantitative image analysis. Data are reported as uncalibrated optical density (n = 17-18 animals). Two animals from the aged insulin aspart group were removed from analysis: one for poor tissue quality, one for failing the visual acuity test. For binding measures in the olfactory bulb (internal plexiform layer), data from one aged saline animal was removed due to poor tissue quality.

2.3.5 Immunohistochemistry

The IHC groups were split as follows: young saline n = 6, young aspart n = 5, aged saline n = 8, aged aspart n = 7. Animals were anesthetized with Fatal-Plus® (390 mg/mL pentobarbital) and perfused with oxygenated saline (~10 min), after which brains were harvested and hemisected. The left hemisphere was placed in 4% PFA for 48 h, then transferred to 30% sucrose for ~24 h. Tissues were placed in an antifreeze solution at 20 °C until sectioning. Tissue slices were cut on a cryostat (35 μm) and probed for IRα using a standard IHC protocol (1° antibodies: Abcam #5500 1:200, 2° antibody: Abcam #150077 1:200; Abcam, Cambridge, MA). Slices were placed on subbed glass slides, covered with DAPI-supplemented mounting medium (#P36966; Invitrogen, Carlsbad, CA), and cover-slipped. A Nikon fluorescent microscope using a spectral analysis camera and Nuance® software (Nuance Communications, Burlington, MA) together with ImageJ was used to quantify percent area of a region of interest (ROI) in the CA1 cell body region. Images were thresholded to identify individual cell bodies. Percent area positively labeled

was determined from the particle size algorithm. Percent areas are reported in hippocampal subsections (*strata pyramidale, radiatum* and *oriens*) obtained from 4-8 animals per group. The same size ROI was used for each subsection across slices. To control for cell density across age and hippocampal subfields, data were normalized to the immunopositive area for DAPI signal in each section. Data presented are derived from the average of two independent scorers.

2.3.6 Hippocampal RNA Extraction and Microarray Analyses

Right hippocampi from 26 animals (young saline n = 6, young aspart n = 5, aged saline n = 8, aged aspart n = 7) were isolated over ice and placed in a -80 °C freezer until processed for RNA extraction. To extract RNA, hippocampi were thawed on ice and homogenized in RiboZol™ Extraction Reagent (#97064-948; VWR®, Radnor, PA). RNA was precipitated with chloroform and isopropanol, then resuspended in a 75% ethanol solution. Following extraction, RNA integrity numbers (RIN) were obtained for each sample using standard protocols (University of Kentucky Genomics Core, Lexington, KY). Mean values for each group were as follows: young saline = 7.32 ± 0.14, young aspart = 7.34 ± 0.15, aged saline = 7.33 ± 0.12, aged aspart = 7.29 ± 0.13. No significant difference in RIN was noted between the groups (2-way ANOVA; $p > 0.5$). Samples were stored in a -80 °C freezer until thawed for microarray analysis (Affymetrix™ rat Clariom™ S Assay; Thermo Fisher Scientific). Gene signal intensities were calculated using the Robust Multiarray Average algorithm at the transcript level and data were associated with vendor-provided annotation information.

2.3.7 Statistical Analysis

Spatial memory results are based on a total of 43 animals. Statistical outliers (> 2 standard deviations from the mean) were excluded from analysis. Immunofluorescent data were filtered using an interquartile range approach. Drug and aging effects on endpoint measures were determined using 2-way ANOVAs with Bonferroni post hoc tests. Significance for all comparisons was set at $p < 0.05$.

2.4 Results

2.4.1 Spatial Learning and Memory

Young and aged animals were able to learn the spatial task and escaped onto the platform with decreasing path lengths across the 3 days of training (young $F_{(2,38)} = 11.3$, $p = 0.0001$; aged $F_{(2,40)} = 17.5$, $p < 0.0001$; **Figure 3.1A**). INI did not alter learning rates in either age group ($p > 0.05$). These results align with our prior work showing that aged animals are capable of learning the task [126, 127, 220]. Insulin aspart, much like detemir, lispro, or glulisine [126, 127], does not show a measurable influence on the learning component. The memory component of the task was investigated with a 24 h probe and 72 h reversal probe following learning for a new platform location. Path length to goal during the 24 h probe revealed significant memory effects from aging ($F_{(1,39)} = 12.5$, $p = 0.0011$), but not from INI ($p > 0.05$, Fig. 3.1B). Similarly, the number of exact platform crossings highlighted a significant aging difference ($F_{(1,39)} = 16.3$, $p = 0.0002$) without an observed INI difference ($p > 0.05$, Fig. 3.1C). We then trained the animals on a new platform location and waited 72 h before probing again. As expected, analysis of this more

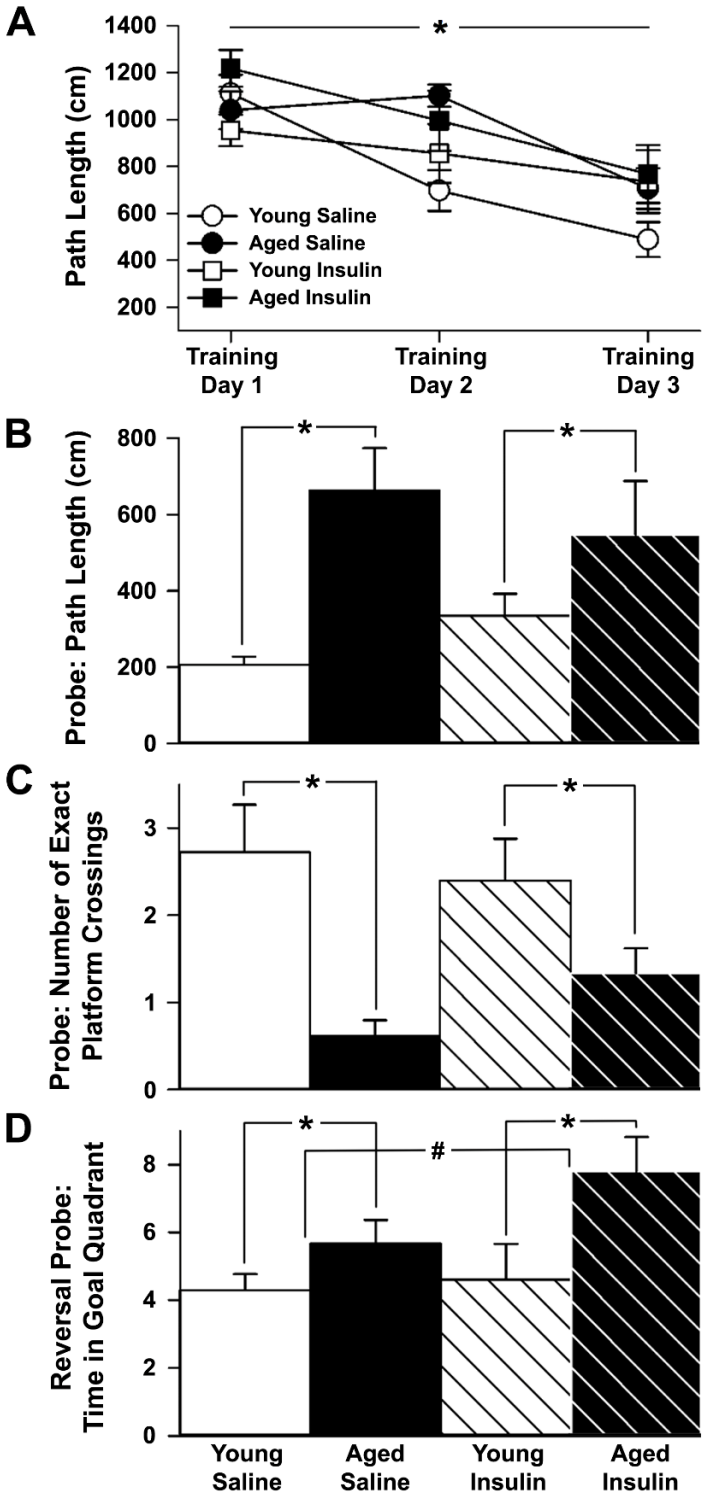


Figure 2.1 Spatial learning and memory. Spatial learning and memory. (**A**) Path length to goal across 3 days of training showed improved learning over time, though no distinct

drug effect was noted. (**B**) Memory recall on the probe task (24h) shows young animals identifying the platform location more readily than aged animals. (**C**) During the full 60s probe task, young animals crossed the exact platform location more often. (**D**) The 72-h reversal probe showed that the young animals spent significantly more time in the new goal quadrant compared to the aged. Further, a trend for INI improving memory regardless of age was noted. Data represent means ± SEM. Asterisks (*) indicate significance at $p < 0.05$. Pound sign (#) indicates a trend at $p < 0.15$.

demanding task revealed significant aging differences on time in goal quadrant during the first 30 s of the probe trial ($F_{(1,39)} = 7.9$, $p = 0.0076$; Fig. 3.1D), but also provided evidence for a trend in improvement in INI-treated animals ($p = 0.14$). While the effect did not reach significance, aged animals showed a ~30% increase in time spent in the correct quadrant while young animals showed no such change. This is likely the reason for a lack of a main effect of INI on ANOVA testing. These results do not appear to depend on swim speed ($F_{(1,39)} = 0.4$, $p > 0.05$, see Methods).

Overall, this INI regimen does not appear to have a greater impact on learning and memory performance in aged animals compared to shorter exposures previously used [126, 127]. While this could suggest that longer exposures are less protective, it could also reflect a short-lived impact of INI that may have been missed using the current protocol. Given that chronic peripheral hyperinsulinemia or insulin resistance can reduce insulin transport into the central nervous system (CNS) [106, 117, 134], we next tested whether 3-month INI could alter CNS IR expression similar to that seen at the BBB [reviewed in 88] or in the periphery.

2.4.2 Quantitative Autoradiography

We harvested brains from randomly selected animals ($n = 5$ per group) to characterize IR binding using autoradiography (**Figure 3.2**). While no significant differences with age or insulin treatment were found in field CA1 of the hippocampus ($p > 0.05$, Fig. 3.2B), a trend for an aging effect in the dorsal blade of the dentate gyrus was noted ($F_{(1,14)} = 3.1$, $p = 0.10$; Fig. 3.2C). Binding of ^{125}I-insulin in the thalamus decreased

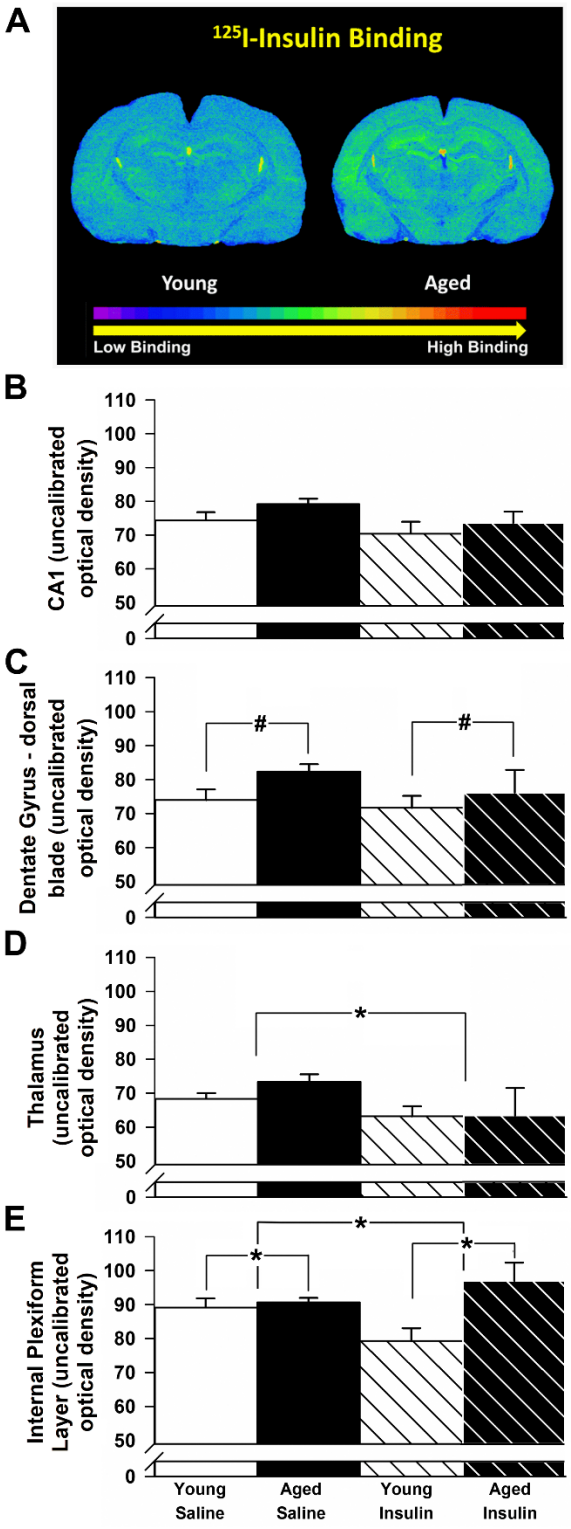


Figure 2.2 ^{125}I-Insulin receptor binding. ^{125}I-Insulin receptor binding. (**A**) Representative images of ^{125}I-Insulin receptor binding on a young and aged control brain section. (**B**) No

significant differences with age or insulin treatment were found in field CA1 of the hippocampus. (**C**) A trend of insulin increasing binding with age in the dentate gyrus was noted. (**D)** Binding in the thalamus decreased significantly with long-term INI. (**E**) Binding in the internal plexiform layer of the olfactory bulb increased significantly with age. A significant interaction term was also noted, with INI decreasing ^{125}I-insulin binding in young while increasing it in aged. Data represent means ± SEM. Asterisks (*) indicate significance at $p < 0.05$. Pound signs (#) indicate trends at $p < 0.10$.

significantly with long-term INI ($F_{(1,14)}$ = 4.7, p = 0.047; Fig. 3.2D), but no aging effects were observed. A significant main effect of age, evidenced by greater binding in the outer plexiform layer ($F_{(1,13)}$ = 7.9, p = 0.014; Fig. 3.2E) together with a significant interaction term in response to INI ($F_{(1,13)}$ = 5.7, p = 0.032; Fig. 3.2E), was noted in the olfactory bulb. These results are somewhat surprising given previous work highlighting decreased cortical IR numbers [218] and overall IR mRNA levels with age [34], but are well-aligned with several studies that did not find significant reductions in IR binding, except in the olfactory bulb of the aged rat [75, 120].

2.4.3 Immunohistochemistry

Aligned with prior work showing decreases in IR mRNA with aging [34], we show a significant reduction in immunolabeled area for IR in field CA1 of the hippocampus in aged animals compared to young (**Figure 3.3**). DAPI signal (% area covered) did not change with age or treatment (**Supplemental Figure 3.1**). A significant reduction in FITC/DAPI was seen in *stratum oriens* ($F_{(1,18)}$ = 4.5, p = 0.047; Fig. 3.3B) and *stratum pyramidale* ($F_{(1,19)}$ = 6.4, p = 0.021; Fig. 3.3D) with age. Interestingly, a trend of increased immunopositive area in response to chronic INI was also observed in *stratum pyramidale* (2-way ANOVA, p = 0.1173; Fig. 3.3D). No significant difference was noted in *stratum radiatum* (Fig. 3.3C). Similar quantification in the dorsal blade of the dentate gyrus did not show a significant main effect of age or INI (p > 0.05; **Supplemental Figure 3.2**).

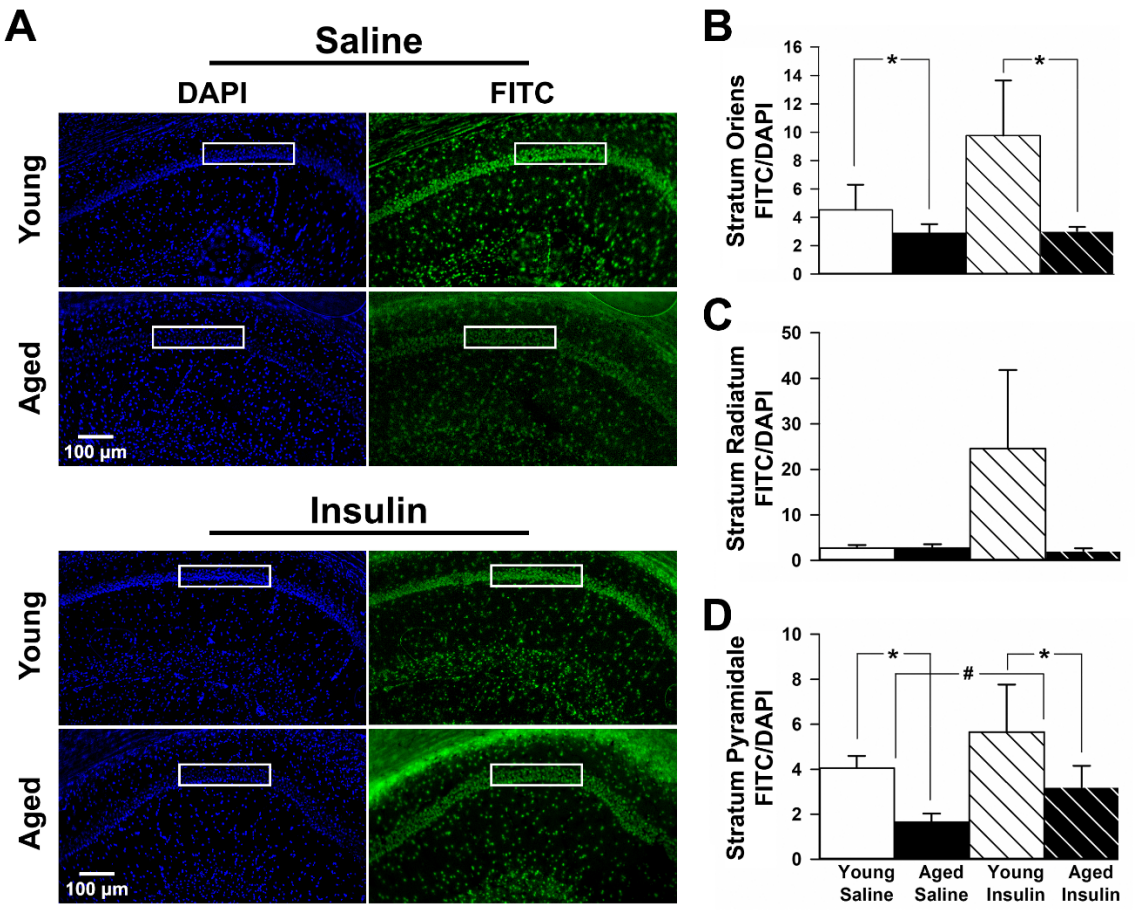


Figure 2.3 IR immunofluorescence. (A) DAPI fluorescence (left) was used to normalize all FITC fluorescence (right) for each hippocampal section quantified. Immunopositive signals representing the presence of the IR were quantified within each ROI (white boxes). Equally sized ROIs were used to quantify immunopositive areas across *strata oriens* (**B**), *radiatum* (**C**), and *pyramidale* (**D**) subfields. *Strata oriens* and *pyramidale* both showed a significant decrease in IR fluorescence with age. *Stratum pyramidale* also showed a trend of increased immunostaining with INI. Data represent means ± SEM. Asterisks (*) indicate significance at $p < 0.05$. Pound sign (#) indicates a trend at $p < 0.12$.

2.4.4 Microarray Analyses

Microarray data are presented in **Figure 3.4**. Of the initial 11,160 filtered genes, significant main effects of age, insulin, and the interaction term identified 1541 genes (2-way ANOVA). We chose a significance level of 0.03 based on the p-value frequency distribution which indicated a large increase in significant genes below that value. As shown in Fig. 3.4A, a greater number of genes were modified by aging (~1100) than by INI (~400).

Transcriptional targets of age and insulin are presented in **Supplemental Table 3.1** (GO Accession *#pending*). A large proportion of genes upregulated by age in the hippocampus combined into functional annotation clusters using the Database for Annotation, Visualization and Integrated Discovery (DAVID) hierarchical clustering analysis, including those involved in neutrophil activation, myelination, inflammation, and cell migration. We validated these changes by testing for alignment with 8 transcriptional profiles of aging published in prior work [221-226, reviewed in 227]. As seen in Fig. 3.4C, aging-significant genes identified here showed a strong correlation with those highlighted by prior profiles (192 genes; p = 1.09E-46; $r^2 = 0.661$). The heatmap representation of the top 10 genes changed with aging or with insulin is shown in Fig. 3.4D. Genes at the intersection of both main effects (age- and insulin-sensitive) are displayed graphically as a function of log2 fold change (Fig. 3.4E). The nearly 140 genes representing the interaction term of the 2-way ANOVA are presented in Fig. 3.4F.

A DAVID heuristic categorization analysis of non-redundant genes upregulated by INI in the hippocampus identified main biological processes that included anti-inflammation (*Synj2bp*), synaptic stabilization (*Pak1, Stx1a*), and tumor

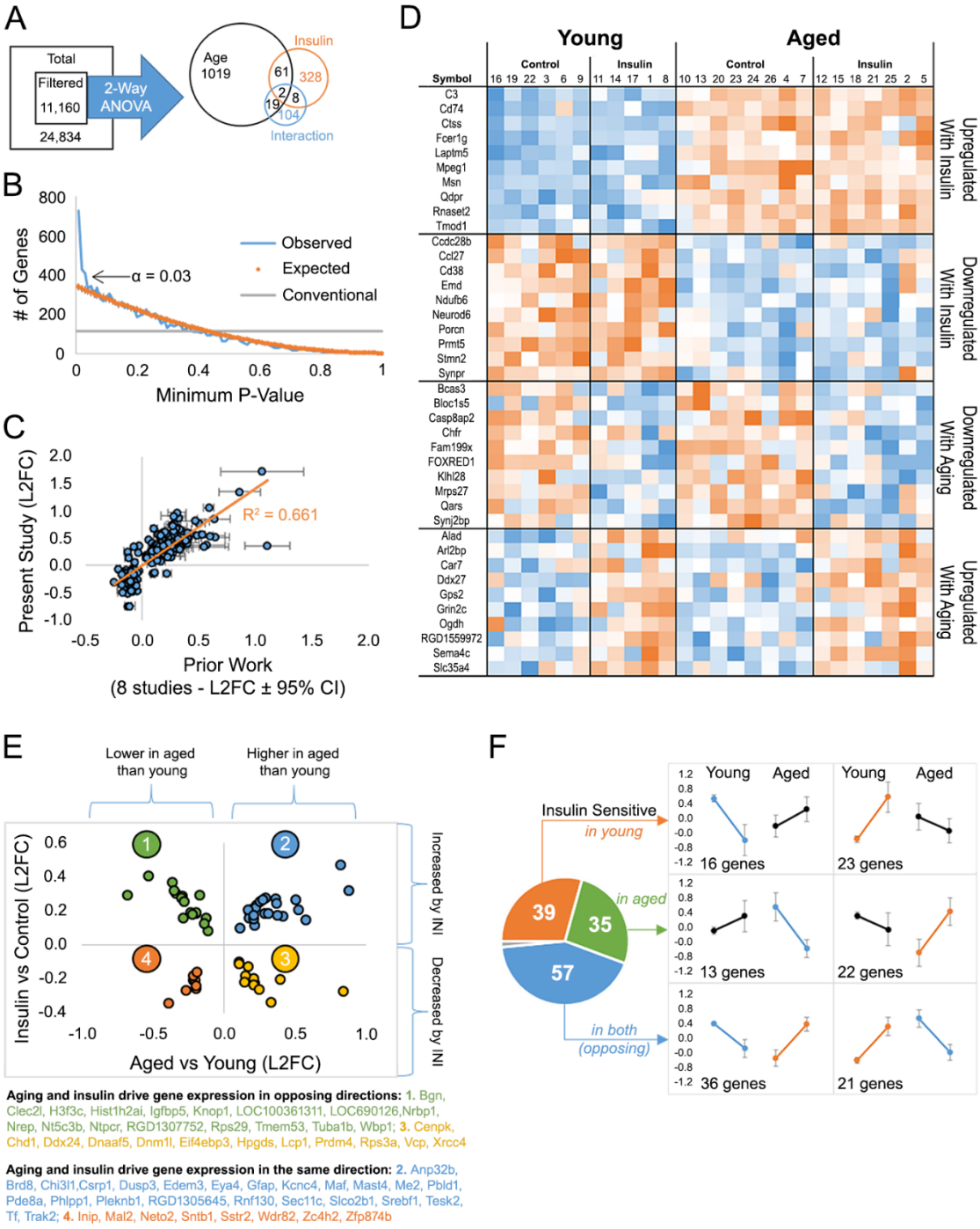


Figure 2.4 Microarray analyses. (**A**) Total gene set filtered to remove low intensity signals yielded 11,160 genes. Two-way ANOVA identified ~1500 genes that were significant by main effects of age, insulin, and/or the interaction. (**B**) P-value frequency histogram shows the increase in the number of significant genes with $\alpha < 0.03$. The

conventional line (gray) delineates a cutoff for significance near 112 as the first percentile ($p < 0.01$) of the 11,160 filtered genes. The orange line represents the p-values obtain when testing for significance across a set of 11,160 randomly generated numbers through a 2-way ANOVA. The blue line highlights the p-values obtain from our dataset. (**C**) We validated the ~1000 age-sensitive genes across our prior studies and found a significant correlation with prior work. (**D**) Heat map of significant genes (top ten) separated across subject by aging or drug effects (lighter colors indicate less change; orange indicates an increase, blue indicates a decrease). (**E**) Genes significant by both main effects (63) fall into four categories: 2 in the same direction (quadrants 4 and 2), and 2 in the opposite direction (quadrants 1 and 3). (**F**) Genes within the significant interaction space (~130) are divided as those modified in young animals (top), in aged animals (middle), or in both (bottom). This result suggests insulin sensitivity in the brain may not differ across aging (39 genes changed in young, and 35 changed in aged). Data represent means ± SEM.

suppression/antiproliferative function (*Cdh11*) genes. RNA signatures downregulated by INI were associated with cancer development (*Erbb2, Myt1*) and glial and neuronal growth (*Atl1, Fgf9, Numb, Acap2*), perhaps providing a path for stabilizing established synaptic connections. Alternatively, the strong presence of *Erbb2* in astrocytes and *Myt1* in oligodendrocytic precursor cells [228] suggest non-neuronal cell types may also be targets of INI. Analysis of genes that responded to aging and insulin in opposite directions, and are therefore likely reparative, corresponded to reduced inflammation, DNA repair, cell growth, and translation stability processes (Fig. 3.4E). Genes increased with age and decreased by INI included two helicases (*Chd1, Ddx24*), a ligase (*Xrcc4*), and a programed cell death gene (*Dnml1*). Those decreased by age and increased by insulin included anti-inflammatory (*Igfbp5*), cellular repair (*Nrep, H3f3c*), vascular function (*Nrep*), tumor suppression (*Nrbp1, Wbp1*), and neuronal growth (*Tuba1b, Nrbp1*) genes. Surprisingly, very few, if any, genes targeted by chronic INI fell within the canonical insulin signaling pathway. It is also interesting that INI altered gene expression similarly in both young and aged animals (35 & 39 genes, respectively), suggesting that the aged hippocampus may remain sensitive to insulin provided the ligand is present. Overall, the profiling analysis presented here reveals that INI likely entered the hippocampus and significantly altered expression of important genes associated with tumor suppression, neurogenesis, and synaptic stabilization.

2.5 Discussion

The present study was undertaken to determine whether INI using a higher-penetrance insulin analogue could prevent aspects of brain aging. Aspart provided an

observable, albeit small, behavioral enhancement during a particularly challenging memory task in the aged animals. A significant reduction in IR autoradiography was seen in the thalamus in response to INI, but not in the hippocampus, although a modest increase in hippocampal IR immunofluorescence in *stratum pyramidale* was noted. Compared to a prior study from our group using fewer insulin exposures (~10), but conducted in young and aged F344 rats with similar doses and INI techniques [126], this much longer study (~63 exposures) did not provide greater improvement on memory recall of spatial information in the aged animals. Importantly, however, we detected significant changes in gene signatures in the hippocampus of INI-treated animals, many of which could represent new therapeutic targets.

2.5.1 Why Insulin Aspart?

The fast-acting insulin analogue aspart includes molecular modifications that increase absorption rates [229-231] and peak plasma concentrations [229] to almost twice that of human insulin. Despite these pharmacokinetic differences, profiles of IR affinity, dissociation and tyrosine kinase activation rates, IGF-I binding, metabolic potency, and ligand degradation rates have been comparable between insulin aspart and human insulin [229, 232]. In the context of INI, insulin aspart's inability to form hexamers may increase its absorption in the brain. In fact, recent work indicated insulin aspart was absorbed more rapidly than longer-acting formulations following nasal delivery in rats [219]. Additionally, INI aspart significantly improved word list recall in healthy male subjects (aged 18-35 years) compared to human insulin [207].

It is reasonable to assume that increasing the availability and absorption rate of insulin could enhance delivery to the brain. While no statistical comparisons between our

prior studies [126, 127] and the current results were performed, insulin aspart does not appear superior at offsetting memory decline in aged animals compared to insulin detemir (longer-acting) or lispro (rapid-acting) [126]. Given similar receptor affinities of the different insulin formulations, and assuming equivalent distribution in the brain, it is possible that the inconsistencies in memory performance in aged INI animals were due to different exposure frequencies. The greater number of exposures (~63 doses, one per day) did not yield larger improvements in memory recall compared to the fewer exposures (~10 doses) used in earlier studies [126]. Interestingly, we previously reported that repeated doses of insulin glulisine (~18, one per day), another rapid-acting insulin, also did not improve memory recall in aged animals, although it did increase hippocampal IR signaling and CBF [127].

Others investigating long-term (30-60 days), repeated dosing of INI in C57BL6/J mice showed that longer exposures are not as beneficial on olfactory or object recognition memory compared to acute [192]. The authors speculated that longer exposures likely initiate a state of brain insulin resistance whereby continued IR signaling is not maintained. While we do not provide evidence of insulin-mediated decreases in IRs in the hippocampus, we do show significant reductions in the thalamus following repeated INI. This could represent evidence for insulin resistance in the thalamus, though it is not clear why this region would be more sensitive to chronic insulin. Whether a larger number of insulin exposures (> 10) weakens its impact or if the formulation used is responsible for this inconsistency remains to be determined. Alternatively, both of these factors could be relevant. Regardless, the evidence of region-specific downregulation of IR presented here

suggests that insulin resistance and/or decreases in signaling may not be a generalizable condition.

Using the SAMP8 mouse model of dementia, 8 weeks of INI improved cognition in earlier stages of the disease (< 24 weeks) but could not ameliorate severe cognitive dysfunction at later stages (24-40 weeks) [190]. In another long-term study (~3 months of INI) conducted in Wistar rats tested at ~11 months of age, learning was improved; however, INI did not improve learning on the MWM task in younger adult animals [201]. This highlights the importance of age in these studies [122, 126, 127, 216, 217] and suggests a need for more analyses in mid-aged animals. Furthermore, current evidence indicates variability in results across labs and conditions may be due to the length of exposure [192], as long-term INI could potentially upregulate insulin degradation and cause a pharmacokinetic tolerance in the brain.

2.5.2 Difference Between ^{125}I-Insulin Receptor Autoradiography and Immunofluorescence Results

Prior reports on IR binding in the brain of rodents indicated higher receptor density in the olfactory bulb and choroid plexus compared to other structures [103, 120, 233]. Additionally, a reduction in IR binding has been noted in the olfactory bulb in studies of aging [75, 120], while others have shown reduced binding in the cortices of elderly non-demented subjects (> 65 years old) compared to younger adults [129]. Contrary to these studies, we do not show reduced IR binding with age in the internal plexiform layer of the olfactory bulb, and instead report greater binding in this area (Fig. 3.2E). Given that insulin levels in the olfactory bulb fluctuate with feeding state [234], it is possible this might have influenced IR autoradiography. It is also interesting to speculate that the exposure to an

enriched environment (e.g. MWM and repeated daily handling) in the current study might have increased IR mRNA levels [114], thereby offsetting the impact of aging on IR density.

In a prior study of adult Sprague-Dawley rats, chronic intracerebroventricular insulin failed to alter IR binding [151]. However, as hypothesized, we show that chronic INI exposure does decrease binding, albeit only in the thalamus. Further, a significant interaction term (2-way ANOVA) showing an increase in aged and a decrease in young animals following INI was noted in the internal layer of the olfactory bulb. It appears distinct brain regions may respond differently to this particular dosing regimen, as areas associated with spatial memory processes (i.e. the hippocampus) were less affected than others. With respect to aging, our results align well with a prior study showing no difference in insulin binding in the cortex or hippocampal formation [120]. Further, data presented here should not reflect IGF-I receptor binding, as an insulin dose well below the binding affinity for IGF-I receptor [12] was used. Thus, our results are novel and given the paucity of binding studies in normal aging, continual investigations of insulin binding in aging and/or AD appear warranted.

Additionally, the strong hippocampal immunofluorescence for the IRα subunit not only reflects the presence of IRs in neurons, but also shows substantial expression in *stratum radiatum* astrocytes and what are likely oligodendrocytes near the heavily myelinated fimbria above *stratum oriens* (Fig. 3.3). While surprising given evidence of stronger immunopositivity in primary neurons compared to other hippocampal cell types [81, 82], this result was corroborated using another IRα antibody from a different company (data not shown), suggesting that IRs in oligodendrocytes are perhaps relevant to observations of neuropathy in DM. Immunofluorescence in the primary neuronal cell layer

was mostly somatic, as no dendritic elements appeared to be immunoresponsive. This was unexpected given previous evidence of synapse-centric insulin effects on hippocampal neurons [reviewed in 90].

Overall, the results from hippocampal IR immunofluorescence do not align well with those from autoradiography. We show a reduction in immunopositive area in *stratum oriens* and *stratum pyramidale* with age that is not reflected in measures of ^{125}I-insulin binding. This is surprising as both approaches report on plasma membrane proteins and should represent functional IRs. Our immunofluorescence protocol did require the use of a mild detergent during washing; thus, it is possible that the quantification reported here includes intracellularly labeled nascent proteins. Further, the area quantified for autoradiography encompassed most of the hippocampus while more defined *strata* were quantified in the immunofluorescence assays, which could have influenced our results. Nevertheless, these results still clearly emphasize the complexity and dynamic aspect of insulin's actions in the brain.

2.5.3 Analysis of Hippocampal Genes Altered by Aging and INI

For the first time, our studies provide a comprehensive analysis of the hippocampal transcriptome in aging that is sensitive to INI aspart. Despite the observed trend towards improved memory in the aged INI group, these gene targets appear to be involved with processes other than those strictly aligned with learning and memory. A number of the genes identified, particularly those modified by aging, are similar to genes characterized in prior studies, including those associated with myelination, inflammation, and cell migration. With respect to genes upregulated by INI, many were also previously recognized in studies of cardiac and brain health, and are primarily involved in anti-

inflammatory [reviewed in 235] and synaptic stabilization processes [reviewed in 90, 236]. Our results offer new evidence that insulin in the brain may have antiproliferative properties and could potentially act as a tumor suppressor. In fact, hippocampal RNA signatures downregulated by INI included *Erbb2*, one of the most well-documented cancer-related genes [237]. Perhaps of greater interest were genes that responded to INI in the opposite direction from aging and are likely beneficial. Again, these centered on processes associated with reductions in inflammation, DNA and cellular repair, and tumor suppression.

Insulin-mediated processes are thought to be reduced in both healthy and pathological aging and are often considered reflective of brain insulin resistance. However, we show that the number of genes altered by INI in the young and the aged brain are nearly identical. Given consistent evidence of maintained insulin sensitivity with age from our group, this is perhaps not surprising. Together, these results suggest that aging may not affect insulin sensitivity as much as previously thought [122].

2.5.4 Conclusion

Aging is the major risk factor for AD. With the expected increase in life expectancy, the resultant growth of the aged population will increase AD incidence; therefore, it is important to consider novel therapies and perhaps earlier interventions for successful brain aging. We report that long-term INI aspart was well-tolerated and present evidence of mild improvements in memory recall in aged animals following repeated daily dosing. We also demonstrate the feasibility of using INI to offset changes associated with brain aging and provide new insights into potential mechanisms and physiological components that may contribute to its therapeutic efficacy.

2.6 FUNDING

This work is supported by the National Institutes of Health ([R01AG033649] to O.T., [T32DK007778] to H.N.F., and [T32AG057461] to A.O.G.).

2.7 ACKNOWLEDGEMENTS

Authors H.N.F, A.O.G., and K.L.A. performed Morris water maze behavioral tests and analyses. Authors H.N.F., A.O.G., and K.L.A. performed animal perfusions and brain extractions. Authors E.S. and J.R.P. performed ^{125}I-insulin receptor autoradiography and data analysis. Authors A.O.G., E.S.J., K.E.H.-S., H.N.F, O.T., and E.M.B. performed RNA preparation and microarray data analysis and interpretation. K.L.A., G.F., K.V., X.M., and L.R.-L. performed and analyzed immunofluorescence assays. Authors H.N.F., O.T., and N.M.P. wrote and compiled the manuscript text and figures. The authors are grateful for the invaluable help provided by Drs. Lawrence D. Brewer and John C. Gant during Morris water maze behavioral characterization.

CHAPTER 3. INSULIN SIGNALING, CALCIUM DYSREGULATION, AND BRAIN AGING

As stated in Chapter 1, the importance of insulin and IR signaling in the brain is well documented, yet the specific mechanisms targeted by this system are still unclear. While our previous study touched on some potential targets of INI, such as hippocampal gene expression or IR binding, elucidating insulin's impact on specific cellular processes is logistically difficult *in vivo*. In light of this, Chapters 4 and 6 will present two studies performed *in vitro* using primary hippocampal cultures, a system that allows for much more nuanced characterizations of IR signaling at the cellular and molecular level. These studies were conducted to investigate two potential mechanisms of action of IR signaling and INI in the hippocampus. The following chapter provides a brief overview of the first mechanism I tested using this culture system: calcium dysregulation.

3.1 The Role of Calcium in the Brain

Calcium enters the bloodstream following its release from bone, where it is then transported throughout the body as dissolved Ca^{2+} ions. A small portion of these ions are stored within cells in organelles such as the sarco-/endoplasmic reticulum and mitochondria [238]. However, in the brain, a significant amount of Ca^{2+} resides in the extracellular space, at concentrations ~10,000-fold higher than that inside the neuron. This large gradient allows for transient changes in intra- and extracellular ion concentrations during neuronal processes and is tightly regulated by ATP-activated ion pumps [238]. The ability of calcium to modulate these important processes relies on voltage-gated calcium

channels (VGCCs), transmembrane proteins responsible for regulating the influx of Ca^{2+} ions from the highly concentrated extracellular space into the interior of the cell.

3.1.1 Voltage-Gated Calcium Channels

VGCCs are transmembrane proteins comprised of polypeptide subunits surrounding a central pore that is primarily permeable to Ca^{2+} ions, although other ions, such as barium, may also pass through [239, 240]. The channels are voltage-gated, meaning that their conformational state (open or closed) depends on the voltage of the surrounding membrane. VGCCs are separated into two broad categories based on their conductance kinetics: high-voltage activated (HVA) and low-voltage activated (LVA). HVA VGCCs activate upon large depolarizations (above -40 mV) whereas LVAs are opened at lower voltages (above -60 mV). The structure of HVA VGCCs consists of 5 polypeptide subunits (α_1, β, γ, and the $\alpha_2\delta$ dimer), while the LVA VGCCs consist only of α_1 and β subunits [239]. HVA and LVA VGCCs can be further differentiated by their subtype, which appears to be determined solely by the α_1 subunit, of which there are 3 major families: Ca_v1, Ca_v2, and Ca_v3.

VGCC subtypes vary in their tissue localization, inactivation profiles, and ion sensitivities [241]. While both HVA and LVA VGCCS can mediate neuronal physiology, most VGCCs in the brain fall into the HVA category, with the predominant subtypes being L-type ("long-lasting" type, containing the α_1 subunit $Ca_v1.2$, $Ca_v1.3$, or $Ca_v1.4$), N-type ("neural" type, containing the α_1 subunit $Ca_v2.2$), and Q- and P-type ("Purkinje" type, containing the α_1 subunit $Ca_v2.1$) [241, 242]. Additionally, these subtypes can also be further differentiated by their subcellular localizations, with N-, Q-, and P-type channels being abundantly presynaptic while L-type VGCCs are predominantly postsynaptic [241].

At resting membrane voltage (-70 mV), VGCCs remain closed, inhibiting extracellular Ca^{2+} from entering the cell. However, as an action potential travels to the terminal of the presynaptic neuron, the shift of the membrane to a more positive voltage triggers the channels to open, allowing Ca^{2+} from the extracellular space to move inside. The influx of Ca^{2+} into the intracellular space then activates numerous cellular processes including synaptic transmission and neurotransmitter release, alterations in gene expression, and hormone secretion. After Ca^{2+} influx, the VGCCs are inactivated, either through prolonged membrane depolarizations or by calcium-mediated processes such as calmodulin-VGCC binding [241, 243].

3.1.2 Neuronal Action Potentials and Synaptic Plasticity

Calcium-mediated processes are an essential component of neuronal function. One such example is rapid, calcium-dependent signal transduction and action potential propagation. In neurons, binding of presynaptic neurotransmitters to receptors on the postsynaptic cell triggers the opening of ligand-gated channels and influx of positive ions (e.g. sodium) into the cytosol which then opens additional channels, resulting in localized, transient depolarizations known as excitatory post-synaptic potentials (EPSPs). EPSPs are graded, meaning that multiple EPSPs concentrated in a single area of a postsynaptic membrane will be additive . The combination of multiple EPSPs in a small region pushes the membrane towards an even more positive charge, thus increasing the likelihood that it will reach its threshold voltage (-55 mV) and fire an action potential.

A presynaptic action potential travels along an axon toward the axon terminal, where it eventually triggers the release of neurotransmitters into the synaptic cleft. This process is regulated by the influx of Ca^{2+} ions from the extracellular space into the cell

interior via VGCCs. Here, the Ca^{2+} ions bind to calmodulin, which in turn activates calmodulin-dependent protein kinase II (CaMKII). CaMKII then phosphorylates synapsin, a neuron-specific protein involved with intracellular docking of neurotransmitter-containing vesicles near the active zones of the synapse [244]. Phosphorylation of synapsin releases the vesicles, allowing them to enter the active zone for subsequent exocytosis and release into the synaptic cleft. The process then repeats as this new set of neurotransmitters binds to the next cell, thus propagating the initial signal from neuron to neuron.

Calcium-dependent CaMKII has been shown to be highly important for synaptic plasticity, especially in the hippocampus [244-246]. Inhibition of CamKII results in diminished learning and memory and impaired long-term potentiation (LTP) [244, 247]. LTP, defined as the process of a particular synapse strengthening over time due to repeated, concurrent activity at both the pre- and postsynaptic neuron [244], is considered the basis for hippocampal memory formation and storage. The calcium-dependent CaMKII has been shown traffic AMPA receptors to the post-synaptic density (PSD) as well as phosphorylate them, which increases their sensitivity to ligand binding and strengthens the synapse, thus aiding in LTP initiation [244]. Additionally, changes in intracellular calcium concentrations also regulate synaptic plasticity by triggering calcium-induced calcium release (CICR) through binding to ryanodine receptors (RyR) at the sarcoplasmic reticulum [248]. The activation of RyR triggers release of calcium from intracellular stores into the cytosol, leading to an amplification of the already rising Ca^{2+} levels within the cell. This additional calcium release appears to be an integral component of calcium-mediated neuronal physiology, as alterations of RyR have been shown to contribute to deleterious

elevations in neuronal calcium levels, increased cell death, and impairments in learning and memory [249-252].

In addition to being a regulator of intracellular processes such as neurotransmitter release and synaptic plasticity, calcium is also an integral component of maintaining and returning neurons to their membrane resting potential. This process involves the opening of calcium-dependent channels, such as potassium channels, which then allow passage of ions into the cell and trigger subsequent hyperpolarization of the membrane [238]. In certain situations, prolonged hyperpolarization may occur. This particular process, referred to as an afterhyperpolarization (AHP), contributes substantially to age- and AD-associated calcium dysregulation and appears to be solely dependent on Ca^{2+} ion-actions [253].

3.1.3 The Calcium-Dependent AHP

As stated in the previous section, Ca^{2+} influx following membrane depolarization and VGCC opening repolarizes the cell to its resting membrane potential by triggering potassium channel activation and K^+ influx, thus resetting it for future depolarizations. However, this process is not exact, and during ion influx, the cell undergoes hyperpolarization in which the membrane voltage "undershoots" the resting potential and falls to voltages below -70 mV. During this refractory period, the cell is unable to generate subsequent depolarizations. Sodium-potassium pumps then redistribute Na^+ and K^+ ions to bring the membrane back to its resting state. The AHP is a calcium-dependent hyperpolarizing event that maintains the hyperpolarization of the refractory period, thus inhibiting neuronal firing. Fast and medium AHPs can occur following a single action potential. However, in the hippocampus, repeated trains of action potentials may sometimes lead to increases in intracellular K^+ that can remain for several seconds after

stimulation [254, 255]. These long K^+ conductances can result in generation of the slow AHP (sAHP), which inhibits further neuronal firing for up to 1 second or more.

Logically, the rate at which the cell is able to recover from the hyperpolarizing refractory period influences the speed at which neurons are able to fire multiple action potentials. Slower recovery from AHP events will naturally lengthen this time period, thus slowing neuronal activity. For this reason, the duration and amplitude of calcium transients in the brain, particularly in the hippocampus, are considered to be important mediators of learning, memory, and neuronal physiology. In fact, alterations in these calcium transients have been implicated in a variety of neurological pathologies. One study using hippocampal slices prepared from young and aged rats indicated the presence of significantly larger AHPs in aged animals compared to young [253]. This observation, along with other early evidence of alterations in calcium-mediated processes during aging and AD, lead to the formulation of a new theory: The Calcium Hypothesis of Alzheimer's Disease and Brain Aging.

3.2 The Calcium Hypothesis of Alzheimer's Disease and Brain Aging

The calcium hypothesis of AD and brain aging was developed in the late 80s and states that dysregulation in neuronal calcium levels leads to cognitive decline by elevating calcium transients, increasing VGCC activity, AHP amplitude, and AHP duration, and disrupting cellular ion homeostasis [256, 257]. Homeostasis of extra- and intracellular calcium levels is an integral part of healthy brain function. Fluctuations in this balance can disrupt the frequency and duration of neuronal activity, alter neurotransmitter release, and increase neuronal cell death [258, 259]. Evidence of age- and/or AD-associated alterations

in calcium homeostasis has been reported from numerous investigators using a variety of different models and techniques. Landfield and colleagues provided some of the earliest data regarding this topic, showing that aging increases VGCC activity, calcium currents, and the amplitude and duration of the calcium-dependent AHP [253, 260-262]. Later work from this same group tied these early observations to aspects of synaptic plasticity [263], suggesting that alterations in calcium-mediated processes may underlie impairments in learning and memory.

Another early study using the F344 animal model of aging showed that aged animals had impaired regulation of Ca^{2+} at nerve terminals, decreased ligand affinity in calcium transporters, and alterations in calcium-activated synaptic ATPase indicative of diminished synaptic transmission [264]. In mice, aging was associated with a decrease in calcium uptake in the brain, which could in turn reduce calmodulin activation and neurotransmitter release [265]. Aged rabbits given nimodipine, an L-type VGCC antagonist, had smaller AHPs, a reduction in the slow phase of the Ca^{2+} action potential, and improved learning [266], providing evidence that targeting calcium dyshomeostasis may be an effective method to ameliorate age-related cognitive decline. Unsurprisingly, early evidence of calcium dysregulation was also reported in AD [267, 268], further strengthening the proposal that it may be an important therapeutic target. More contemporary studies have since supported these initial findings [269-272], while also reporting that age- and/or AD-related calcium dysregulation is associated with alterations in intracellular calcium-related processes [249, 269] and synaptic plasticity [252, 273, 274], can contribute to cell death and excitotoxicity [250, 275], and that targeting this dysregulation can improve learning, memory, and synaptic function [276-278].

Interestingly, calcium dysregulation has also been linked to aspects of peripheral metabolism [271]. In fact, rats rendered severely or moderately diabetic through STZ-administration have worsened performance on the MWM as well impaired LTP compared to controls [59]. STZ-induced diabetes has also been shown to increase resting intracellular calcium concentrations and reduce depolarization amplitudes in neurons [279], echoing prior findings in the aged and AD brain. Larger sAHPs and elevated spike broadening have also been detected in the CA1 region of STZ-induced diabetic rats [280]. Similarly, diabetes was shown to elevate resting calcium levels and reduce calcium mobilization and intracellular calcium release in the STZ rat model [251]. Recent work from our lab has provided some contradictory results regarding this relationship, as we reported that short-lived diabetes in young-adult ZDF rats did not exacerbate markers of age-related calcium-dysregulation [281]; however, the substantial evidence of calcium dysregulation in aging and AD, its reported connection to T2DM, and the impairment of brain IR signaling in these same disease states, strongly suggests that insulin may play a role in the regulation of calcium homeostasis.

3.3 Combating Neuronal Calcium Dysregulation

3.3.1 Insulin and Calcium-Dependent Processes

An early study in rat pinealocyes showed that insulin could reduce VGCC activity, providing the basis for its use as a therapeutic in targeting age-related calcium dysregulation [282]. Since then, additional evidence of insulin's ability to impact calcium-related processes in the brain have been reported. A study performed on retinal slices from salamanders also showed that insulin inhibited VGCC currents as well as decreased Ca^{2+}

influx following depolarization [283]. In primary rat hippocampal neurons, insulin inhibited spontaneous Ca^{2+} oscillations in a process driven by MAPK-activation [284]. Insulin has also been shown to induce long-term depression (the opposite of the memory-forming LTP process) at hippocampal mossy fiber-CA3 and CA1 synapses by elevating intracellular calcium concentrations and L-type VGCC activity in postsynaptic neurons through the PI3K/PKC-dependent trafficking of AMPA receptors [92, 285].

Prior work in our lab has also suggested that insulin can impact calcium-related processes. In a study using hippocampal slices obtained from young and mid-aged F344 rats, we showed for the first time that acute exposure to exogenous insulin significantly reduced the calcium-mediated AHP, providing a direct link between IR signaling and calcium dysregulation [55]. Further, rats maintained on HFD for 4.5 months lost this insulin response, suggesting that peripheral metabolic dysfunction, such as diabetes, not only impacts insulin activity but also calcium-mediated processes, at least in the hippocampus. Interestingly, this study also showed that slices from mid-aged animals had a greater reduction in the AHP than their younger counterparts, again providing evidence that insulin resistance in the aged brain may not be due to receptor desensitization, but rather a lack of available ligand (see Chapter 1, section 1.3.4). In another study, we again presented evidence of insulin's ability to reduce markers of calcium dysregulation by reporting that insulin administration to hippocampal slices significantly reduced the amplitude and duration of the sAHP in Sprague-Dawley rats (2-6 months old) and produced a trend for a reduction in the duration and amplitude of both the medium and sAHP in young and aged F344 rats [126]. Finally, our lab also showed that acute insulin administration to cultured, primary hippocampal neurons resulted in reduced VGCC

currents and Ca^{2+} transients [204]. Together, this evidence strongly supports the role for insulin and IR signaling in neuronal calcium processes.

3.3.2 Targeting Calcium Dysregulation in Cultured Hippocampal Neurons Using a Molecular Approach

While work from our lab, as well as from other groups, has investigated calcium-related processes following administration of insulin [55, 126, 204] or similar ligands, such as IGF-I [286], the direct effects of elevated insulin signaling on VGCC activity remain unclear. As INI has been shown to have substantial positive effects on learning and memory in aging and AD, and because calcium dysregulation has also been identified as a contributor to these pathologies, we proposed the hypothesis that impaired insulin signaling in aging and AD increases hippocampal calcium dysregulation and that elevating IR signaling attenuates this dysregulation, thus providing a mechanism for INI's beneficial effects.

However, whether conducted *in vivo, ex vivo,* or *in vitro*, most studies of IR-associated processes have been performed using exogenous insulin administration, which could introduce confounds such as the ligand binding to other receptors (i.e. IGF-I receptors), activation of IR-mediated metabolic pathways, or alterations in yet unknown cellular mechanisms that may be sensitive to rising insulin concentrations. In light of this, I performed a study directly investigating insulin's ability to reduce VGCC activity in neurons by using a constitutively active form of the human IR to elevate signaling while bypassing potential variables associated with exogenous ligand administration. This modified receptor, known as IRβ, was produced by truncating a significant portion of the α subunit of the human IR, which then confers constitutive activity to the catalytic βsubunit

[287]. After expressing this receptor in primary hippocampal neurons, I then measured VGCC activity using whole-cell patch clamp electrophysiology. This work is one of the few measures of direct, IR-mediated VGCC activity in this cell type, and is presented in the following chapter.

The following manuscript has been published in Neurochemical Research: Neurochem Res. (2019). 44: 269. Epub 23 March 2018. doi.org/10.1007/s11064-018-2510-2. I performed all cell culture preparations, lentiviral infections of cell cultures in a BSL2 laboratory, protein harvest and Western immunoblot assays, immunocytochemistry and photomicroscopy, whole-cell patch-clamp electrophysiology experiments, and data analyses. Author(s) S. D. Kraner designed and oversaw production of the IRβ receptor; G. J. Popa and M. D. Mendenhall constructed all plasmids and lentiviruses used in these experiments as part of the University of Kentucky Genetic Technologies Core; K. L. Anderson assisted with Western immunoblots; K. K. Hampton performed confocal imaging during immunocytochemistry experiments. In this study, I sought to explore the hypothesis that elevated IR signaling could attenuate markers of age-related calcium dysregulation in cultured hippocampal neurons by reducing voltage-gated calcium channel currents. The constitutively active human IR, IRβ, was chosen to bypass potential confounds associated with use of the ligand (e.g. binding of insulin to other receptors, such as IGF-I) by conferring elevated receptor activity in the absence of exogenous insulin. While prior studies had tested the effects of elevated insulin signaling on calcium-related processes *in vitro* using administration of insulin, to our knowledge, no study had used a purely molecular method to directly observe the outcome of chronic, constitutive IR signaling on calcium channel activity.

CHAPTER 4. EXPRESSION OF A CONSTITUTIVELY ACTIVE HUMAN INSULIN RECEPTOR IN HIPPOCAMPAL NEURONS DOES NOT ALTER VGCC CURRENTS

H. N. Frazier[1], K. L. Anderson[1], S. Maimaiti[1], A. O. Ghoweri[1], S. D. Kraner[3], G. J. Popa[2], K. K. Hampton[1], M. D. Mendenhall[2], C. M. Norris[3], R. J. Craven[1], and O. Thibault[1]

[1]Department of Pharmacology and Nutritional Sciences, University of Kentucky Medical Center, UKMC, 800 Rose Street, Lexington, KY 40536, USA

[2]Department of Cellular and Molecular Biochemistry, University of Kentucky Medical Center, UKMC, 741 S. Limestone, Lexington, KY 40536, USA

[3]Sanders-Brown Center on Aging, University of Kentucky Medical Center, UKMC, 800 S. Limestone, Lexington, KY 40536, USA

4.1 ABSTRACT

Memory and cognitive decline are the product of numerous physiological changes within the aging brain. Multiple theories have focused on the oxidative, calcium, cholinergic, vascular, and inflammation hypotheses of brain aging, with recent evidence suggesting that reductions in insulin signaling may also contribute. Specifically, a reduction in insulin receptor density and mRNA levels has been implicated, however, overcoming these changes remains a challenge. While increasing insulin receptor occupation has been successful in offsetting cognitive decline, alternative molecular approaches should be considered as they could bypass the need for brain insulin delivery. Moreover, this approach may be favorable to test the impact of continued insulin receptor signaling on neuronal function. Here we used hippocampal cultures infected with lentivirus with or without IRβ, a constitutively active, truncated form of the human insulin receptor, to characterize the impact continued insulin receptor signaling on voltage-gated calcium channels. Infected cultures were harvested between DIV 13 and 17 (48 h after infection) for Western blot analysis on pAKT and AKT. These results were complemented with whole-cell patch-clamp recordings of individual pyramidal neurons starting 96 h post infection. Results indicate that while a significant increase in neuronal pAKT/AKT ratio was seen at the time point tested, effects on voltage-gated calcium channels were not detected. These results suggest that there is a significant difference between constitutively active insulin receptors and the actions of insulin on an intact receptor, highlighting potential alternate mechanisms of neuronal insulin resistance and mode of activation.

4.2 INTRODUCTION

Insulin signaling in the brain is an integral physiological component of proper neurological function and has been shown to help maintain receptor trafficking (AMPA, NMDA, $GABA_A$) [288-292], increase cerebral blood flow [127, 176, 178, 179, 199], stimulate glucose transporter translocation [152, 293], reduce voltage-gated calcium channel (VGCC) function [204, 282, 283], reduce neuroinflammation [154], and reduce ryanodine receptor function [204]. Studies on age-related alterations in insulin signaling have highlighted a reduction in insulin receptor (IR) density and IR mRNA in aged brains [34, 121, 129, 294]. Intranasal insulin (INI) administration has been shown to improve cognitive function in both young and aged individuals [164, 170, 171, 177, 182, 184, 210] with similar reports in animal models of Alzheimer's disease (AD) and aging [87, 154, 189, 281, 295]. While the mechanism by which insulin exerts these physiological effects is not fully understood, some evidence suggests that it may be related to calcium signaling.

Both classic and contemporary evidence suggests that tight regulation of intracellular calcium levels is required for normal cellular function [270, 296-302]. In response to evidence of neuronal calcium dysregulation in aging, the calcium hypothesis of brain aging was developed [256, 257]. This hypothesis states that calcium dysregulation can lead to cognitive decline by increasing calcium transients, VGCCs, and calcium-mediated afterhyperpolarization (AHP) [261, 263, 273, 303, 304]. Our lab has shown that insulin administration leads to a reduction in the slow-AHP in rat hippocampal neurons [55, 281, 295]. Together, this evidence highlights a possible connection between insulin signaling and calcium homeostasis with regards to age-related cognitive decline. These data also suggest that maintaining insulin signaling is a viable therapeutic approach to

address this decline [55, 126]. Indeed, others have used administration of a chemical supplement (oxaloacetate) to increase insulin signaling in the brain [305]. Based on these findings, we sought to explore the impact of molecular enhancement of IR signaling in the absence of exogenous insulin by expressing a truncated, constitutively active human IR (IRβ) in rat primary hippocampal neurons.

IRβ is a modified human IR consisting almost solely of the catalytic β subunit of the human IR [287]. This truncation leads to insertion into the plasma membrane together with constitutive activity of the receptor in mouse fibroblasts. Here we tested the hypothesis that expressing a modified, constitutively active form of the IR in neurons would increase insulin signaling without the need for exogenous delivery of insulin, and would reduce VGCC currents in hippocampal neurons. We infected mixed primary hippocampal cultures with two lentiviral constructs: synapsin-IRβ-dTomato and synapsin-IRβ-mCherry and their respective controls. Each construct consists of a neuronal specific promoter (synapsin) and a fluorescent reporter gene (dTomato or mCherry). Cells were either harvested for protein analysis or recorded using whole-cell patch-clamping methods to quantify VGCC currents 4-7 days following infection. Results show that while constitutive activity was obtained, there was no evidence of changes in VGCC current density. This result is surprising, given the acute and robust effects of exogenous insulin on VGCC and ryanodine receptor function in hippocampal neurons previously reported by our lab [204]. Ongoing studies are investigating the ability of hippocampal neurons to maintain insulin signaling across time to better define mechanisms of insulin insensitivity in the brain, i.e., down-regulation or desensitization of receptors, together with down-stream signaling pathways.

4.3 METHODS

4.3.1 Cell Culture

Hippocampal mixed (neuron/glia) cultures were prepared as described previously [203, 275, 306] and established from E18 Sprague-Dawley rats. E18 fetuses and hippocampi were dissected under a microscope in ice-cold Hank's balanced salt solution (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 4.2 mM $NaHCO_3$ and 12 mM HEPES (pH 7.3). Hippocampi were transferred to 0.25% trypsin EDTA solution at 37 °C (Thermo Fisher Scientific) and left at room temperature (23 °C) for 11 min. Trypsin was removed and the hippocampi were washed three times with SMEM (Minimum Essential Medium supplemented with 200 mM L-glutamine (Thermo Fisher Scientific) and 35 mM D-glucose). Hippocampi were then titrated and diluted with SMEM to the desired final concentration (100,000 neurons/mL) before being plated in 2 mL aliquots onto 35 mm plastic dishes (Corning Inc., Corning, NY) that were previously coated with 10 mg/mL poly-L-lysine (1 h) for a final cell density of 200,000 cells per dish. Cultured neurons were incubated (37 °C, 5% CO_2, 95% O_2) for 24 h before the first medium exchange, when half of the medium was replaced with 90% SMEM and 10% horse serum (Thermo Fisher Scientific). After 3 days *in vitro* (DIV), half of the media was replaced with SMEM containing horse serum, 5-fluoro-2-doxyuridine, and uridine to stop glial cell growth.

All experiments were conducted following a 24 h exposure to a no serum, low glucose (5.5 mM) MEM to maintain normal glucose oxidation rates and insulin sensitivity [203]. Insulin time-course treatments were performed using 10 nM insulin glulisine diluted

in sterile saline (Apidra®, stock solution of 100 U; Sanofi-Aventis, Bridgewater, NJ). As a control for our insulin time course experiments, either a 5 or 30 min saline exposure was used to normalize the data. No significant difference we seen between 5 and 30 min control treatments. All data presented were obtained at room temperature (23 °C).

4.3.2 Lentiviral Construction and Infection

EcoRI sites and the human IRβ protein was ligated between the XbaI and BamHI sites using PCR and standard digestion protocols. The synapsin-IRβ-mCherry plasmid was constructed using an pHR-SFFV-KRAB-dCas9-P2A-mCherry vector (gift of Jonathan Weissman, Addgene plasmid #60954). The self-cleaving P2A site preceding the mCherry sequence produces mCherry expression at a 1:1 ratio with the IRβ protein, thus improving fluorescence. The synapsin promoter and human IRβ protein were ligated into the vector between the AscI and BamHI sites, replacing the Cas9 sequence via PCR and standard digestion protocols. All segments constructed using PCR were sequenced to verify fidelity. All plasmids were then converted into lentiviruses by co-transfecting HEK293 cells with the donor plasmid, PsPAX2, and pMD2.G (gifts from Dr. Didier Trono, Addgene plasmids #12260 and #12259) using a polyethyleneimine (80 µg/ml, nominal MW 40,000, pH 7) and NaCl (75 mM) mixture to induce uptake of the DNA. Culture supernatants were withdrawn over a 5-day period, clarified by centrifugation, and the virus precipitated with polyethylene glycol (1.4% w/v) and NaCl (50 mM). The viral pellet was resuspended in cold PBS. Lentiviruses were stored at -80 °C until needed. Viruses were then thawed on ice and immediately administered to culture dishes. mCherry expression was monitored using a Nuance spectral analysis camera with wavelengths above 510 nm (CRi, Inc.,

Boston, MA). Phase and fluorescence photomicrographs were overlaid in Adobe Photoshop.

4.3.3 Protein Harvest and Western Blots

Mixed primary hippocampal cultures were lifted in RIPA buffer containing phosphatase and protease inhibitors. Cells were further lysed using polytron agitation. Protein levels were quantified using a BCA assay and a microplate reader. Western blots were used to quantify differences in protein expression. Samples were run in duplicate within and across gels and were averaged. Proteins were assessed with the following antibodies: AKT #4685S 1:1000 and pAKT #4051S 1:1000 (Cell Signaling Technology Inc., Danvers, MA). Blots were developed with chemiluminescence and digitally imaged on a scanner (G-Box; Syngene, Frederick, MD). Gray values were obtained using the ImageJ gel analysis tool (Version 1.46r; Wayne Rasband, National Institutes of Health, Bethesda, MD). For each blot, mean gray value of pAKT and AKT were normalized to a saline-treated sample, and pAKT was divided by AKT to generate ratios.

4.3.4 Immunocytochemistry

Primary hippocampal cultures, uninfected or infected with syn-IRβ-dTomato lentivirus, were fixed using 4% paraformaldehyde (PFA) in 1X PBS for 20 min. Immunocytochemistry was performed using a primary antibody targeted to the HA-tag present on our truncated IRβ protein (HA-Tag #3724S 1:1600, Cell Signaling Technology) in conjunction with a fluorescent secondary antibody (Alexa Fluor® 488 #A-11070 1:200, Thermo Fisher Scientific). Cultures were imaged using a spectral camera (Nuance FX, CRi, Inc.) and a FITC dichroic mirror equipped with a long-pass emission filter (>525 nm). A

series of images were acquired from 490 to 650 nm and were used to define the green signal and all other fluorescent signals (autofluorescence and background noise). The green channel was extracted from the total fluorescent signal using the Nuance algorithm (spectral library subtraction).

4.3.5 VGCC Recording Solutions

For whole-cell recordings of VGCC currents, the external solution was prepared as follows (in mM): 111 NaCl, 5 $BaCl.H_2O$, 5 CsCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 20 $TEACl.H_2O$. The solution was brought to pH 7.35 with NaOH and 500 nM tetrodotoxin (TTX) was added before recording to inhibit Na^+ channels. The internal pipette solution was prepared as follows (in mM): 145 MsOH, 10 HEPES, 3 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 13 $TEACl.H_2O$, 14 phosphocreatine Tris-salt, 4 Tris-ATP, 0.3 Tris-GTP. The solution was brought to pH 7.3 with CsOH. All solutions were sterile filtered using a 0.22 μm vacuum filter (Corning).

4.3.6 Whole-Cell Recording and Analysis

All electrophysiological data were acquired between DIV 13 and 17, 4 to 7 days post-infection. 1.5 mm glass whole-cell patch-clamp electrodes (Drummond Scientific, Broomall, PA) were made using a P-87 micropipette puller (Sutter Instruments, Novato, CA). The culture dish was rinsed with recording solution three times, then filled with 3 mL of the extracellular solution supplemented with 500 nM TTX. To allow for currents to stabilize, all data were recorded 3 min after the whole-cell configuration was achieved. *IV* (current-voltage) relationships (-60 to +30 mV) were initially conducted to identify maximal current voltage. In Figures 2 and 3, an increase in current density is seen between

the *IV* data and the current density data presented in bar graph form. This is likely because *IV*s were conducted 3 to 5 min prior to peak VGCC current measures. Cells were held at -70 mV and currents were elicited (150 ms) at the maximal peak response derived from the *IV*. All currents were leak subtracted using 5-8 scaled hyperpolarizing sub-pulses. Because insulin may alter cell size, we report on measures of current densities (pA/pF) derived from dividing maximal current amplitude (average of 5 depolarizations taken 30 s apart) by membrane capacitance (measured in pCLAMP™) for each cell. All electrophysiological data were collected between 4 and 7 days post-lentiviral infection. All recordings were conducted on the stage of an E600FN microscope (Nikon Inc., Melville, NY) placed on an anti-vibration table. An Axopatch 1D (Molecular Devices, Sunnyvale, CA) in combination with a digidata 1200 AD board and pCLAMP™ 7 (Molecular Devices) were used for electrophysiology acquisition. Data were digitized at 5-10 KHz, low-pass filtered at 2-5 KHz, and were quantified in Clampfit 7 (Molecular Devices).

4.3.7 Statistical Analysis

Electrophysiological results are based on a total of 76 hippocampal neurons obtained from the pups of 6 pregnant dams. Statistical outliers (>2 standard deviations from the mean) in each data set were excluded from further analysis. Transgene effects on endpoint measures were determined with unpaired t-tests and ANOVAs, and Bonferroni post hoc tests, when necessary. Significance for all comparisons was set at $p < 0.05$.

4.4 RESULTS

4.4.1 Western Blot Analysis

We used Western blot techniques to quantify the ratio of pAKT/AKT in mixed hippocampal cultures treated 5-30 min with 10 nM insulin (**Figure 4.1A and B**). By 15 min of activation a trend for an increase in signaling was noted ($n = 3$; one-way ANOVA $p = 0.06$), and by 30 min, the pAKT/AKT signal was significantly elevated compared to 30-min saline controls ($n = 3$; $p < 0.05$). Elevated levels of pAKT/AKT at 15 and 30 min confirm continued IR activity at these time points and suggest signaling in neurons increases with ligand exposure time.

To test for constitutive activity of the truncated, human IRβ receptor in the absence of exogenous insulin, we infected hippocampal cultures (DIV 6-8) with EF1a or EF1a-IRβ lentiviruses. Cells were harvested for Western blot 48 h post-infection to allow adequate time for protein expression. Cells expressing IRβ showed significantly elevated pAKT/AKT compared to cells infected with the EF1a negative control (Fig. 4.1C and D; $n = 3$; t-test $p < 0.005$). Compared to the ligand-derived 30-minute time point (Fig. 4.1B), the increase in signaling was smaller, nearly reaching a 3-fold increase at 48 h. Thus, compared to control conditions, the IRβ receptor was expressed and yielded an increase in activity. The results also confirm that the infection protocol resulted in constitutive activity in the absence of added insulin.

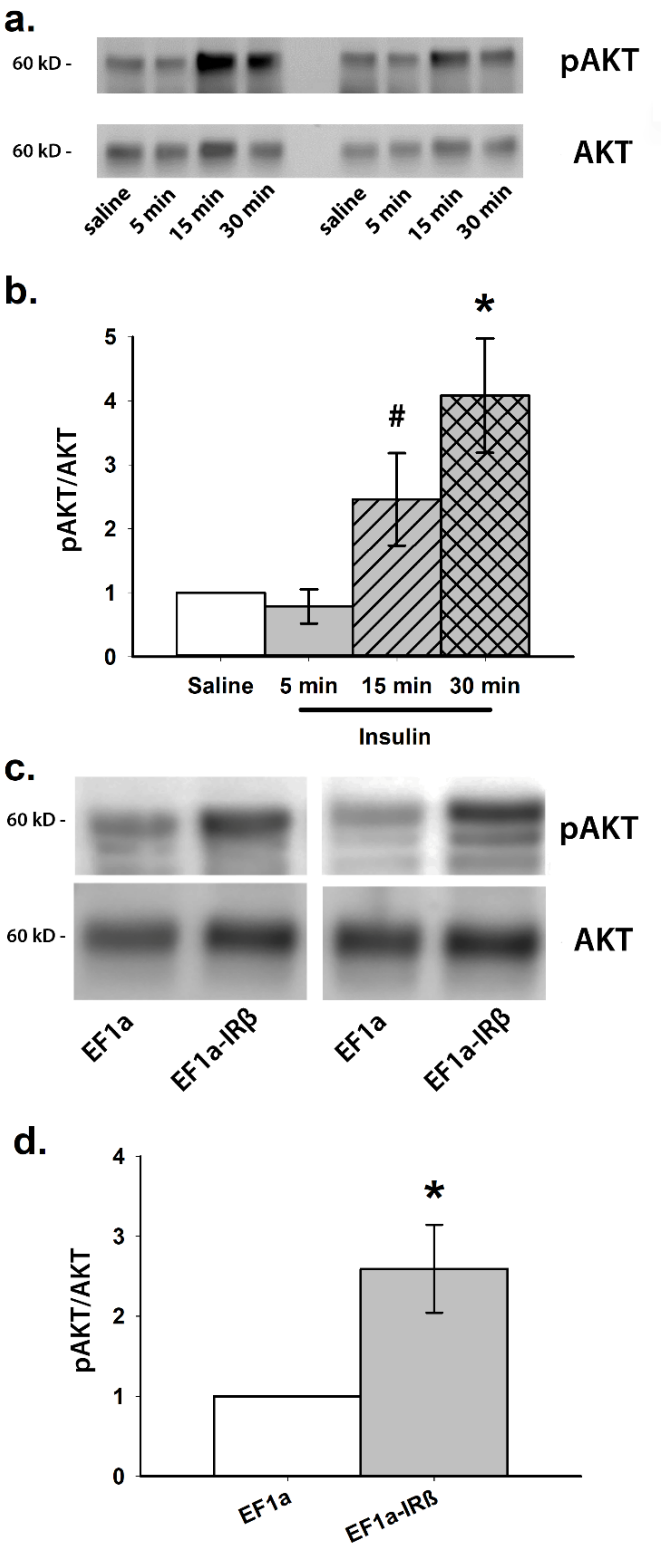

a.
60 kD -
pAKT
60 kD -
AKT
saline
5 min
15 min
30 min
saline
5 min
15 min
30 min
b.
pAKT/AKT
0
1
2
3
4
5
#
*
Saline
5 min
15 min
30 min
Insulin
c.
60 kD -
pAKT
60 kD -
AKT
EF1a
EF1a-IRβ
EF1a
EF1a-IRβ
d.
pAKT/AKT
0
1
2
3
4
*
EF1a
EF1a-IRß

Figure 4.1 Insulin signaling with and without exogenous insulin. (a.) Representative Western blots of mixed primary hippocampal cultures treated with saline or 10 nM Apidra® for 5, 15, or 30 min. Each sample was run in duplicate and probed separately across gels. Blots were probed with Cell Signaling Technology anti-phospho AKT (Ser473; #4051) 1:1000 and total AKT (pan #4685) 1:1000. **(b.)** Quantification reveals signaling increases after 15 and 30 min compared to timed saline controls (n = 3). **(c.)** Representative Western blots of mixed primary hippocampal cultures infected with Ef1a or EF1a-IRβ lentiviruses. Each sample was run in duplicate and probed separately across gels. Blots were probed as in b. **(d.)** Western blot quantification reports significant pAKT/AKT between EF1a and EF1a-IRβ, suggesting constitutive activity (n = 3). Pound sign (#) indicates $p < 0.10$. Asterisks (*) indicate $p < 0.05$. All data represent means ± SEM.

4.4.2 Electrophysiological Analyses of the IRβ Construct Containing IRES and dTomato

To test whether elevated insulin signaling altered VGCC currents, we performed whole-cell patch-clamp experiments on hippocampal neurons infected with either the negative control (syn-dTomato) or IRβ-containing lentiviruses (syn-IRβ-dTomato) (**Figure 4.2A**). Cultures were placed in a low glucose, no serum growth media for 24 h prior to electrophysiology recordings on days 4-7 post-infection. Prior to electrophysiological experiments, cultures infected with syn-IRβ-dTomato were fixed for immunocytochemistry staining to confirm IRβ expression using the HA reporter tag present on the IRβ protein. Anti-HA fluorescent antibody indicated successful expression of IRβ in approximately 80% of neurons (Fig. 4.2B). Live pyramidal neurons were patched and passive membrane properties were recorded from a holding potential of -70 mV. Neither cell capacitance, holding current at -70 mV, nor membrane resistance were found to be different (**Table 4.1**). For each cell recorded, we then determined the voltage necessary to elicit maximal current amplitude using an *IV* protocol (-60 to +30 mV). *IV* recordings (Fig. 4.2C and D) from negative control and IRβ-expressing neurons were averaged and compared between groups (n = 30 per group). No significant difference in VGCC current threshold or peak voltages were seen between groups (Fig. 4.2D; one-way ANOVA with Bonferroni post hoc $p > 0.05$).For each neuron, VGCC activity generated during the maximum activation voltage step was measured at three different time points: i.e., at peak activity (peak), during the last 10 ms of the voltage step (late), and 50 ms after the voltage step (tail) (Fig. 4.2E). Current activity at each time point was statistically comparable in neurons expressing IRβ or dTomato control (n = 30 per group; two-way

Table 4.1 Cellular and electrode parameters

	synapsin-dTomato	synapsin-IRβ-dTomato	uninfected	synapsin-IRβ-mCherry
Membrane Capacitance (pF)	71.90 ± 3.60	66.10 ± 3.53	59.36 ± 3.95	67.46 ± 5.78
Membrane Resistance (MΩ)	450.30 ± 45.34	555.20 ± 42.48	528.90 ± 95.84	322.79 ± 39.11
Access Resistance (MΩ)	11.60 ± 0.86	11.10 ± 0.69	9.87 ± 1.20	9.13 ± 0.50
Holding Current (pA)	-131.00 ± 14.35	-98.29 ± 13.56	-86.77 ± 12.08	-147.94 ± 22.93

Table 4.1 Cellular and electrode parameters. Data represents means ± SEM obtained from four different groups of cells (n = 78) studied under patch-clamping conditions to record VGCC currents. No significance was detected between the groups.

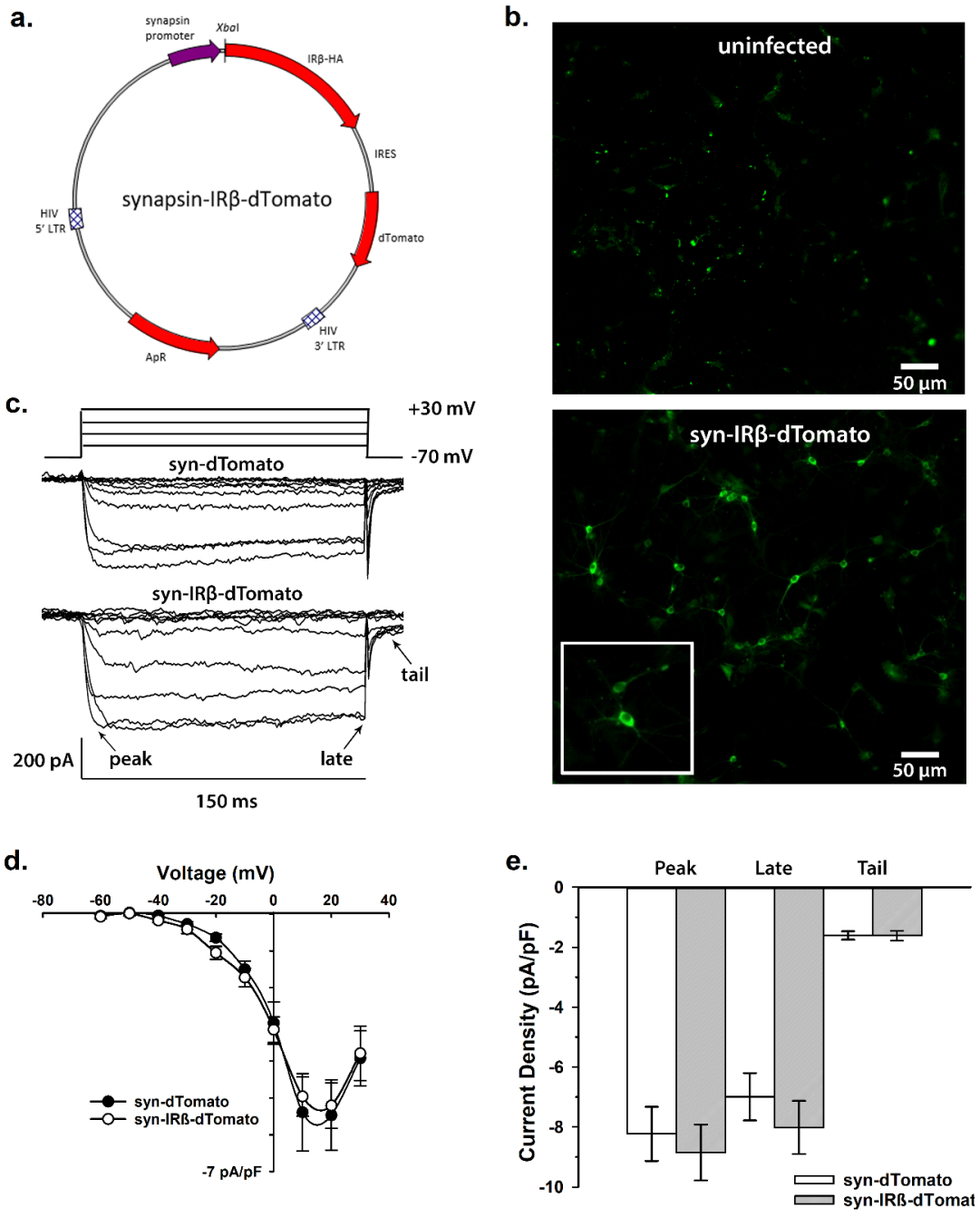


Figure 4.2 Constitutive activity of the human truncated IRβ subunit does not alter voltage sensitivity of VGCCs. (a.) Plasmid map of synapsin-dTomato construct. The IRβ sequence was inserted between XbaI and BamHI sites using PCR ligation for production of the synapsin-IRβ-dTomato plasmid. **(b.)** Photomicrograph of hippocampal neurons

probed for IRβ expression using a fluorescent HA-tag antibody. Cells in green indicate presence of IRβ. **(c.)** Representative inward currents obtained from a holding potential of -70 mV during determination of *IV* relationships (-60 to + 30 mV). **(d.)** Quantification of VGCC currents across groups showed no significant difference. **(e.)** Current density (pA/pF) of peak, late, and 50 ms tail currents were not altered by production of the constitutively active form of the human brain IR. All data represent means ± SEM.

ANOVA with Bonferroni post hoc $p > 0.05$). This indicates that neither maximal flux through VGCCs, nor the number of available channels, nor the inactivation or deactivation rates were affected by constitutive insulin signaling. Note that current recordings were performed 3-5 min after recording of the *IV* to allow the cell to stabilize. This is likely the reason for the small increase in currents reported between *IVs* (Fig. 4.2D) and maximal currents (Fig. 4.2E). To isolate L-type VGCC currents from currents arising from other VGCC subtypes, cells were held at -40 mV for 3 min to inactive N- and T-type channels. The membrane voltage (Vm) was then stepped to the voltage necessary to elicit maximal current amplitude (data not shown). Under these conditions, the presence of IRβ still had no statistically significant effects on peak, late, or tail current activity.

4.4.3 Electrophysiological Analyses of the IRβ Construct Containing P2A and mCherry

Because the IRES sequence does not always drive equal expression of the constitutive active IRβ subunit with the reporter gene (dTomato) [307, 308], we constructed another plasmid using an P2A site and mCherry as the reporter gene (**Figure 4.3A**). This approach yielded more reliable expression of the red fluorescent protein and allowed us to test a second IRβ-expressing plasmid, therefore providing a more thorough characterization of the impact of sustained insulin signaling on VGCCs. For this series of experiments and because mCherry conferred a higher level of fluorescence compared to dTomato we compared IRβ-expressing neurons (red) to uninfected (dark) neurons in the same field-of-view (Fig. 4.3B). Pyramidal neurons were patched and peak currents were derived following the same *IV* protocol previously described (Fig. 4.3C). *IV* recordings from control (uninfected) and IRβ-expressing (syn-IRβ-mCherry) neurons were averaged and compared. No significant difference in *IV* trace recordings was seen between these two

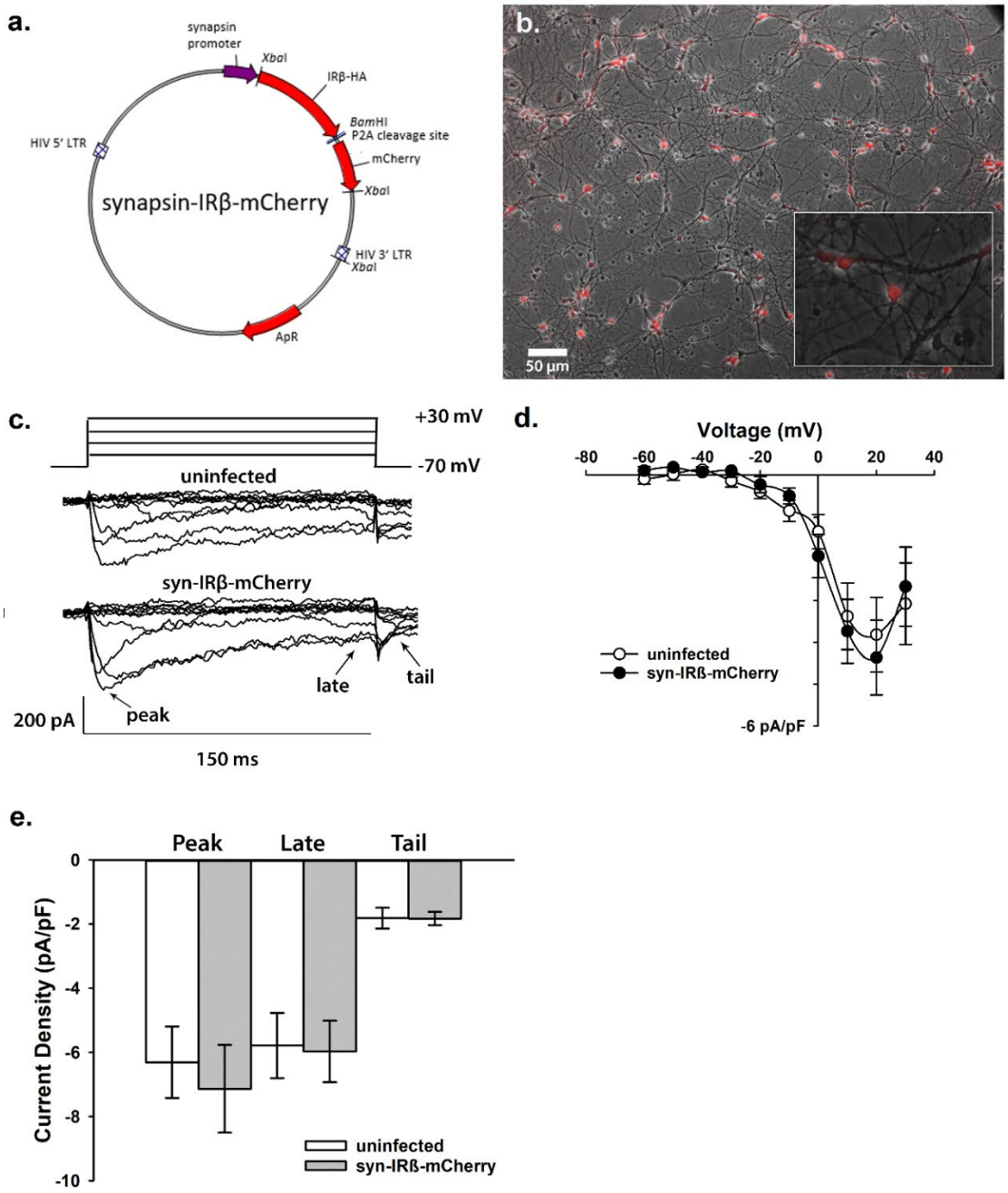


Figure 4.3 A second constitutively active form of the human truncated IRβ subunit does not alter voltage sensitivity of VGCCs. (a.) Plasmid map of synapsin-mCherry IRβ subunit construct. Note replacement of the IRES sequence with the P2A site. The IRβ

sequence was inserted between XbaI and BamHI sites using PCR ligation for production of the synapsin-IRβ-mCherry plasmid. **(b.)** Photomicrograph of cultured neurons exposed 48 h to the synapsin-IRβ-mCherry, we estimate ~70-80% infection efficacy. Uninfected cells (dark) where used as controls. Inset shows both fluorescent and non-fluorescent cells. **(c.)** Representative inward currents obtained from a holding potential of -70 mV during determination of *IV* relationships (-60 to + 30 mV). **(d.)** Quantification of VGCC currents across groups shows no significant difference. **(e.)** Current density (pA/pF) of peak, late, and 50 ms tail were not altered by production of this second constitutively active form of the human IR. All data represent means ± SEM.

groups of cells (Fig. 4.3D; n = 9 per group; one-way ANOVA with Bonferroni post hoc $p > 0.05$). Analysis of maximal currents at peak, late, and tail were then averaged for each cell type. Current recordings from primary hippocampal neurons expressing IRβ did not show a significant difference compared to uninfected controls at any time point (n = 9 per group; two-way ANOVA with Bonferroni post hoc $p > 0.05$). As in Fig. 2.2, VGCCs recorded from a holding potential of -40 mV to increase participation of L-type VGCC, also were not changed by treatment and no significant differences were detected between uninfected and IRβ-expressing neurons at any time point (*data not shown*).

4.5 Discussion

The original intent of this study was to circumvent the need for the ligand at the IR by expressing a constitutively active form of the human IR in hippocampal neurons. The lack of protein quantification from cells infected with either synapsin-containing vectors (Fig. 4.2 and 4.3) prevents us from comparing VGCC effect size between these two conditions (synapsin-IRβ-dTomato versus synapsin-IRβ-mCherry). This is not a major concern given the lack of an overall effect on VGCC. Further, because IRES-dependent expression of the downstream gene (reporter gene) can be significantly lower than the protein of interest [307, 308], we switched to a P2A-dependent vector to confer comparable levels of expression of both gene products. Even with strong mCherry expression (Fig. 4.3B), red cells showed no significant differences when compared to dark cells on measures of VGCC properties. Nevertheless, we show here that viral delivery of a truncated, human IR (IRβ) increased signaling through pAKT/AKT in hippocampal neurons. Interpretation of these data highlights potential interplays between insulin

signaling and calcium homeostasis in neurons, and may also provide clues about intracellular markers used as reporters of insulin sensitivity in neurons.

4.5.1 Why Study Long-Term Insulin Receptor Activation in Neurons?

We and others have shown that acute applications of insulin can reduce VGCC function [204, 282, 283] as well as ryanodine receptor function within minutes [204]. Given that VGCCs and calcium-induced calcium release (CICR) participate in the generation of the AHP [252, 266, 309-311], and that larger calcium-dependent AHPs are seen in neurons from aged, cognitively impaired animals [253, 263, 278], our initial work used repeated daily applications of INI to restore calcium homeostasis and redress cognitive decline in aged animals [127, 281]. However, because neuronal IR signaling can last for extended periods of time, we used electrophysiological techniques to characterize VGCC function in hippocampal neurons following 3-7 days of constitutive IR activity. We sought to identify a novel therapeutic approach to maintain calcium homeostasis by providing constitutive insulin signaling.

Results indicate that expressing three different IRβ-containing plasmid constructs and their controls in neurons raised downstream signaling from the IR for at least 72 h, yet VGCC currents were not affected, even 7 days post-infection. This result is surprising given our previous work showing that acute insulin administration in hippocampal neurons can reduce calcium-sensitive functions. Potential explanations for these results include, but are not limited to, the impact of insulin signaling duration and the activation of different downstream signaling pathways.

4.5.2 Is Time Important?

In contrast to insulin signaling in the periphery where activation is quickly terminated by internalization of the IR in muscle and fat cells [15, 312-314], neuronal IRs can signal for long periods of time without evidence of down-regulation [86, 287]. While we present evidence of long-term (72 h) IR signaling via IRS/PIP_3/AKT, these results suggest that continued activation of this pathways does not reduce VGCCs in neurons. Further, we recently showed that reductions in neuronal calcium levels and calcium-mediated potentials were not seen in the ZDF rat even following a 7-week period of sustained peripheral hyperglycemia and hyperinsulinemia [281]. Additionally, activation of the PI3K/mTOR/AKT pathway was shown to rapidly increase synaptic protein levels within minutes [315], while other pathways, such as MEK/ERK, a pathway which has nuclear targets, have been implicated in modulating the expression of calcium-sensitive channels [316]. It follows that targeting nuclear factors would be slower and likely longer-lasting compared to pathways involved with acute IR activation. Based on the evidence presented here, we propose that signaling pathways other than AKT must exist in neurons to alter long-term calcium homeostasis.

An alternative interpretation is that the molecular approach was successful at reducing VGCCs but only transiently, and at an earlier time than tested here. Unfortunately, the acute impact of lentiviral delivery on VGCCs cannot be determined because of the time constraints associated with changes in protein expression. Standard lentiviral protocols require an incubation period of at least 24-48 h before adequate expression of the protein is reached. Thus, these experimental protocols did not allow us to test for VGCC changes within the same time frame as acute insulin exposures (i.e. 10 min). Given the long-lasting

nature of insulin signaling in neurons, it is clear that future studies are needed to investigate the impact of time on several IR pathways.

4.5.3 What Is Neuronal Insulin Resistance?

Evidence for insulin resistance in neurons has been derived from a multitude of molecular experiments showing reductions in signaling from the level of the IR and IRS1 to GLUT4 [25, 137, 317-320]. While inhibition at any point in this cascade negatively impacts insulin signaling, it is not clear which single point best reflects the phenomenon described as insulin resistance; despite this, focus has historically been placed on AKT. Our evidence of maintained pAKT signaling in the absence of detectable changes in VGCC suggests that observation of AKT phosphorylation by itself may not be a representative indicator of insulin sensitivity in neurons. Additionally, because multiple proteins within the insulin signaling pathway are also sensitive to other agents and cross signaling, analysis of one single aspect of the IR signaling cascade does not specifically assess insulin resistance in the tissue. Therefore, alternative methods for quantifications of insulin sensitivity perhaps need to be considered.

We have used direct insulin administration to test insulin sensitivity in hippocampal slices of young and aged animals. In these studies, the impact of exogenous insulin has repeatedly been greater in aged compared to young neurons [55, 281]. Further, using magnetic resonance spectroscopy (MRS) and cerebral blood-flow data, we also show a greater impact of insulin in aged compared to young brains [127]. Acute application of the anti-diabetic drug pioglitazone on hippocampal slices also provides evidence for greater sensitivity of the drug in aged animals compared to young [220]. Evidence from other groups shows reductions in blood-brain barrier insulin transport may be responsible for the

aged-dependent reduction in insulin sensitivity [128]. In this paper, the author presents evidence that phosphorylation of AKT in aged mice treated with intracerebroventricular injections of insulin is comparable to that seen in young mice. Finally, in animal models of AD, a greater increase in hippocampal IR signaling was seen in mid-age compared to young mice [125]. Together, these data suggest that the underlying insulin sensitivity and the definition of this sensitivity in neurons needs further clarification.

4.5.4 Future Directions and Conclusions

It is clear a more detailed characterization of insulin resistance in neurons is needed in order to better define new and targetable therapies. With respect to the potential impact of insulin and its neuroprotective role in neurons (i.e. reducing calcium influx), much remains unknown, and it is unclear whether the short-acting PI3K pathway or the likely longer pathway through ERK is involved. Also, attention to the subcellular compartmentalization of the modified insulin pathway with aging and AD should be considered. This is likely important, given the evidence that IRs concentrate at synaptic sites [112, 236, 321], and a greater focus on post-synaptic densities where crucial insulin-sensitive ion targets are located should be considered [322].

Overall, it appears we have identified a neuronal model of insulin resistance in the presence of increased pAKT activation. While neuronal insulin insensitivity has been proposed as a contributor to age-related cognitive decline, the mechanisms behind this are not well understood. Specifically, further studies are needed to characterize downstream cellular targets of neuronal IR activation (e.g. glucose utilization, glucose transporters, calcium transporters, calcium buffers, ER calcium homeostasis and others), and to provide

a fuller picture of neuronal insulin resistance with age. Greater definition of IR desensitization, internalization and the mechanisms involved in down-regulation of IR signaling in neurons need investigation in animal models of aging.

4.6 FUNDING

We acknowledge the National Institutes of Health for sources of funding for these experiments: Thibault, O. (R01AG033649); Frazier, H. and Hampton, K.K. (T32DK007778).

4.7 ACKNOWLEDGEMENTS

The authors acknowledge the use of facilities in the University of Kentucky Center for Molecular Medicine Genetic Technologies Core. This core is supported in part by National Institute of Health Grant Number P30GM110787.

CHAPTER 5. INSULIN AS A POTENTIAL REGULATOR OF NEURONAL GLUCOSE METABOLISM

5.1 GLUCOSE METABOLISM IN THE BRAIN

The results from our study of elevated IR signaling and VGCCs in hippocampal neurons was discouraging, as well as surprising, given our prior data of insulin's impact on the AHP and calcium transients in similar models. However, attenuating calcium dysregulation associated with aging and AD is just one of many possible mechanisms behind insulin and INI's beneficial effects on learning and memory in the clinic. Another hypothesis that is rapidly gaining traction in our field is that brain IR signaling, much like in the periphery, elevates energy metabolism by mediating the uptake and utilization of glucose, potentially by increasing GLUT activity. In this chapter, I will present evidence supporting this hypothesis and provide justification for our latest study investigating insulin's impact on glucose metabolism in the hippocampus.

5.1.1 Glucose Transport in the Brain

The brain is a highly metabolically active organ and is heavily (perhaps even solely) reliant on glucose for energy, with some studies indicating that it accounts for up to 20% of total body O_2 consumption in adults and up 50% of total body O_2 consumption in children [323]. Unlike some cells that can undergo gluconeogenesis, such as hepatocytes in the liver, neurons cannot produce their own glucose and are therefore dependent on transport of the sugar from other areas of the body [324]. Glucose passes from the brain microvascular network to the interstitial fluid through the BBB, where it is then taken up by cells in the CNS. Due to being polar and hydrophobic, glucose molecules are incapable of passively moving across the lipid bilayer on their own; instead, their uptake relies on

facilitated diffusion by uniporter transmembrane proteins known as GLUTs [324]. Briefly, free glucose binds to the extracellular binding site of the protein, which then undergoes a conformational change of its C-terminal domain that deposits the sugar on the intracellular side of the membrane [325].

Inside of cells, the sugar undergoes glycolysis, where it is converted into pyruvate following a series of phosphorylation and oxidation events. At this point, the metabolic pathway branches: in the presence of oxygen, pyruvate is completely oxidized through the cellular respiration pathway, producing NADH and CO_2, whereas in anaerobic environments, it is as reduced through the process of fermentation, resulting in NAD^+. While initial studies suggested that glucose metabolism in the brain was entirely aerobic, we now know that some cells, particularly astrocytes, can switch to anaerobic glycolysis during higher cognitive demand [326, 327].

5.1.2 Structure, Function, and Localization of Brain-Specific Glucose Transporters

GLUTs are uniporter proteins comprised of 12 helical transmembrane segments [324, 328]. In humans, there are currently 14 known GLUT subtypes (10 of which have been identified in the CNS) that can be distinguished by their tissue localizations, binding kinetics, regulatory mechanisms, and activation/inactivation pathways [324]. It is believed that the majority of this variation arises from the NH^{2-} and $COOH^-$ terminals and the intracellular loop of the proteins, all of which differ in length and sequence between subtypes [329, 330]. Unlike IR isoforms, which are encoded by the same gene and alternatively spliced to produce distinct functional proteins, GLUT isoforms are encoded by separate genes that are dispersed within and/or across different chromosomes [329]. As the genetic sequences and sizes of these subtypes differ (from the 8 kb *GLUT4* to the

35 kb *GLUT1*), so too do their amino acid sequences, with the smallest (GLUT3) having 496 amino acids and the largest (GLUT2) having 524 [329]. While the number of currently identified, distinct mammalian GLUTs is 14, there are 5 subtypes that could be considered the most well-characterized: GLUT1, GLUT2, GLUT3, GLUT4, and GLUT5. Of these 5, GLUTs 1 and 3 are the isoforms most commonly associated with the CNS; however, GLUTs 2, 4, and 5 have also been detected in various brain regions and cell-types, particularly in astrocytes and oligodendrocytes (GLUT2), microglia (GLUT5), and hippocampal neurons (GLUT4) [324, 331].

GLUT1 is considered the primary BBB GLUT. It is widely expressed on both the luminal and abluminal membrane of the BBB endothelial cells, as well as throughout the brain microvasculature, and is responsible for glucose transport from the bloodstream into the CNS [324, 331]. Interestingly, GLUT1 has also been discovered in astrocytes; however, astrocytic GLUT1 is much smaller (45 kDa) than the heavily glycosylated BBB GLUT1 (55 kDa), suggesting that their functional and kinetic properties are somewhat different. The regulation of GLUT1 is still not fully clear; however, it appears that trafficking and membrane insertion of GLUT1 may be dependent on the general metabolic state and level of circulating glucose [332, 333], the presence of cytokines and growth factors [332, 334-337], and hypoxic stress [338, 339]. Additionally, some work has reported that GLUT1 expression can also be stimulated by insulin [340], and that this mechanism may even rely on the same signaling molecules (e.g. AKT/GSK3β) that are involved with trafficking of the insulin-sensitive GLUT4 in the periphery [341].

While GLUT1 is the primary transporter responsible for initial uptake of glucose into at the BBB, GLUT3 is the isoform predominantly involved with uptake into individual

neurons [324, 328, 329, 331, 341]. Indeed, GLUT3 is often referred to as the "neuron-specific" GLUT [342, 343], and is widely expressed throughout nearly all brain regions [344-347]. In neurons, GLUT3 appears to be localized to the synaptically-dense neuropil and dendritic processes [348-351], providing strong evidence that this particular GLUT is heavily involved with synaptic transmission. GLUT3 has also been shown to have a higher affinity for glucose than many other isoforms [344], which may be explained by the brain's high metabolic demand. As with GLUT1, regulation of GLUT3 expression and trafficking to the plasma membrane appears to be based on glucose and oxygen availability [332, 338, 352, 353], hormones and oxidative stress [354-357], and neuronal activation [358, 359]. In the 90s, several studies reported that GLUT3 expression was upregulated following insulin administration in myotubes and muscle cells [337, 340, 360], yet it is only recently that GLUT3 expression was reported to respond to insulin or insulin-related processes in neurons [52, 361-363]. Additionally, GLUT3 expression appears to be downregulated by aging, AD, and peripheral metabolic dysregulation [324, 364-366], phenotypes that also have reduced IR signaling.

GLUT4 is considered the primary "insulin-sensitive" GLUT and is predominantly found in muscle and adipose tissue [329]. In the periphery, GLUT4 is responsible for initiating uptake of glucose into adipose and muscle cells following the binding of insulin to peripheral IRs. The specific signaling pathways and regulatory processes governing GLUT4 expression/trafficking in the periphery were discussed previously (see Section 1.2.2 and 1.2.3) and will not be elaborated on here. Recently, however, it has become clear GLUT4 is not only expressed in these peripheral tissues, but also in the CNS, albeit at much lower levels [324, 331]. Specifically, these GLUTs appear to be localized to

hippocampal and cerebellar neurons. The expression of GLUT4 in the hippocampus is intriguing, as it suggests that this transporter may have a functional role in hippocampal learning and memory processes. Studies investigating this hypothesis have strengthened this theory, with one reporting that exogenous Aβ administration reduces neuronal GLUT4 localization and impairs LTP [367], and another showing that hippocampal GLUT4 expression is elevated after a learning task and that inhibiting GLUT4 impairs memory acquisition [368]. The impact of aging, metabolic dysfunction, and biomarkers of AD on GLUT3 and GLUT4 expression strongly supports a role for glucose transport and metabolism in cognitive decline associated with these phenotypes.

5.2 Insulin Resistance and Impaired Glucose Metabolism in the Brain

5.2.1 Impact of Aging and AD on Brain Metabolism

As the CNS is heavily reliant on glucose for proper function, it is logical to assume that in situations of suboptimal brain function, such as age- and AD-related cognitive decline, impaired metabolism would also be occurring as well. Recent evidence suggests that this assumption is correct [369]. In the clinic, AD is associated with reduced glucose uptake and utilization [139, 211, 370] which appears to correlate with disease severity and APOE genotype [371-373]. Studies of post-mortem tissue from AD patients have indicated the presence of decreased glycolytic flux and GLUT3 expression that worsened with the severity of the disease [364]. Importantly, some work has also reported that AD-associated perturbations in brain glucose metabolism may occur before the onset of clinical symptoms, implying that metabolic impairment can actually impact the development and progression of the disease [364, 374, 375]. Animal models support these clinical findings,

with APP/PS1 transgenic mouse models having reduced glucose tolerance and early alterations in glucose metabolism [376, 377], similar to that of AD patients.

In addition to AD-specific cognitive decline, alterations in glucose metabolism have also been associated with other forms of dementia and with traumatic brain injury [194, 378-382]. In aging, aerobic glycolysis is reduced, suggesting that the metabolic profile of the aging brain is different than that of the young [383]. Peripheral dysfunction is also associated with altered brain metabolism [380, 384, 385], as middle-aged T2DM patients have been reported to have reduced cerebral glucose usage compared to controls [386]. Additionally, prediabetic and diabetic patients with insulin resistance (based on HOMA-IR scores) presented with reduced cerebral glucose metabolic rate on measures of FDG-PET [38]. Once again, these clinical reports are supported by studies performed in animal models, as well as in cell culture, as diabetes reduces the expression of GLUT4 in the rodent brain [387] while chronic insulin administration *in vitro* decreases acute cellular glucose uptake [317].

5.2.2 IR Signaling as a Potential Target for Elevating Glucose Metabolism

It has been suggested that impairments in CNS glucose metabolism could be mediated by a reduction of insulin and IR-associated processes [370], and indeed, models that have historically presented with impaired brain IR signaling, such as HFD animal models, have also been shown to have reduced GLUT3/4 expression, decreased glucose metabolism, and impaired synaptic plasticity [362]. Therefore, targeting reductions in glucose metabolism by elevating IR signaling may be of therapeutic interest. Recent studies have investigated this approach with promising results. In a rat model of TBI, INI increased glucose uptake, improved memory, and reduced neuroinflammation and hippocampal

lesion volume [194]. Two other studies performed in rats showed that INI ameliorated cerebral hypometabolism, astroglia activation, and neuronal loss in the hippocampus while also reducing Aβ levels [116, 187]. Intrahippocampal insulin administration improved memory and hippocampal glycolysis [293] while ICV insulin was reported to stimulate hippocampal GLUT4 translocation, likely through a PI3K-dependent pathway [152]. Clearly, the impact of impaired glucose metabolism on the aging- and AD-brain cannot be denied, nor can its relationship to IR signaling in the CNS. However, the specific cellular mechanisms used by insulin to influence these metabolic processes is still not fully understood.

5.3 Testing the Effect of Sustained IR Activation on Neuronal Glucose Metabolism in Hippocampal Cell Culture

As stated previously, most studies of insulin actions on hippocampal glucose metabolism were performed by delivering insulin directly into cell culture. While our prior study on the impact of constitutive insulin activity on VGCCs was disappointing, the molecular techniques employed proved to be a novel and effective approach for increasing IR signaling without the need for exogenous ligand administration. The successes in this previous study are highlighted by the high rate of neuron-specific IRβ expression, the lack of any observable neurotoxic effects following lentiviral infection or sustained receptor activation, and a clear, detectable increase in downstream signaling markers, such as pAKT, in the IRβ-expressing cells compared to controls. For this reason, we chose to continue using this system to test the hypothesis that increased IR signaling would enhance measures of glucose uptake and utilization while elevating the expression of GLUT3 and

GLUT4 in primary hippocampal neurons. This study, which provides robust evidence of insulin's ability to impact hippocampal energy metabolism, marks the completion of my dissertation project and is presented in the following chapter.

The following manuscript has been submitted for publication in the Journal of Biological Chemistry. I performed all cell culture preparations and lentiviral infections in our BSL2 laboratory, all protein extractions and Western immunoblot assays, all 2-NBDG imaging recordings, and all data analyses associated with these experiments. Author R. J. Craven performed and analyzed all of the tritium-labeled glucose uptake assays in hippocampal cell cultures. Author(s) S. D. Kraner contributed to the design and production of the IRβ receptor; G. J. Popa and M. D. Mendenhall constructed all plasmids and lentiviruses used in these experiments as part of the University of Kentucky Genetic Technologies Core; L. P. Reagan kindly provided us with the GLUT4 antibody used during Western immunoblot assays; N. M Porter provided substantial help during the manuscript editing and revision process. In this study, I sought to explore the hypothesis that elevated IR signaling could improve neuronal glucose metabolism by increasing glucose uptake and rate of utilization in hippocampal neurons via the upregulation of GLUT3/4 expression and/or translocation to the plasma membrane. The constitutively active IRβ receptor was chosen to bypass potential confounds associated with exogenous insulin administration (e.g. binding of insulin to other receptors, such as IGF-I). While previous studies have tested the effects of elevated IR activity on cellular metabolism and related processes *in vitro* using direct delivery of insulin, to our knowledge, no prior study had utilized a purely molecular method to directly observe the outcome of chronic, constitutive IR signaling on glucose metabolism in cultured hippocampal neurons.

CHAPTER 6. ELEVATING INSULIN RECEPTOR SIGNALING USING A CONSTITUTIVELY ACTIVE HUMAN INSULIN RECEPTOR INCREASES GLUCOSE METABOLISM AND EXPRESSION OF GLUT3 IN CULTURED HIPPOCAMPAL NEURONS

H. N. Frazier[a], A. O. Ghoweri[a], K. L. Anderson[a], R.-L. Lin[a], G. J. Popa[b], M. D. Mendenhall[b], L. P. Reagan[c], R. J. Craven[a], and O. Thibault[a]

[a]Department of Pharmacology and Nutritional Sciences, University of Kentucky College of Medicine, 800 Rose St., Lexington, KY 40536, USA

[b]Department of Cellular and Molecular Biochemistry, University of Kentucky College of Medicine, 138 Leader Ave., Lexington, KY 40506, USA

[c]Department of Pharmacology, Physiology and Neuroscience, University of South Carolina School of Medicine, 6311 Garners Ferry Rd., Columbia, SC 29209, USA

6.1 ABSTRACT

Insulin signaling is an integral component of healthy brain function, with evidence of positive insulin-mediated alterations in synaptic integrity, cerebral blood flow, inflammation, and memory. However, the specific pathways targeted by this peptide remain unclear. Previously, our lab used a molecular approach to characterize the impact of insulin signaling on voltage-gated calcium channels and has also shown that acute insulin administration reduces calcium-induced calcium release in primary hippocampal neurons. Here, we explore the relationship between insulin signaling and glucose metabolism using similar methods. Mixed, primary hippocampal cultures were infected with either a control lentivirus or one containing a constitutively active human insulin receptor (IRβ). 2-NBDG imaging was used to obtain indirect measures of glucose uptake and utilization. Other outcome measures include Western immunoblots of GLUT3 and GLUT4 on cytosol and total membrane subcellular fractions. Glucose imaging data indicate that neurons expressing IRβ show significant elevations in uptake and rates of utilization compared to controls. As expected, astrocytes did not respond to the IRβ treatment. Quantification of Western immunoblots show that IRβ is associated with significant elevations in GLUT3 expression, particularly in the total membrane subcellular fraction, but did not alter GLUT4 expression in either fraction. Our work suggests that insulin plays a significant role in mediating neuronal glucose metabolism, potentially through an upregulation in the expression of GLUT3. This provides further evidence for a potential therapeutic mechanism underlying the beneficial impact of intranasal insulin in the clinic.

6.2 Introduction

The brain, once thought to possess no circulating insulin or functional insulin receptors (IRs), has now been identified as an insulin-sensitive, and perhaps even insulin-dependent, organ. Indeed, insulin signaling in the brain is not only associated with normal healthy brain function and development [74, 88], but is also directly involved with important cognitive processes such as memory and learning [60, 114, 144, 154, 321]. Additionally, a reduction in insulin binding, receptor density, and IR signaling, particularly in the hippocampus, has been associated with aging, Alzheimer's disease (AD), and mild cognitive impairment [31, 75, 120, 121, 129, 131, 134, 388]. Therapeutic approaches aimed at offsetting these impairments by increasing the amount of available insulin in the brain have recently been developed with great success. Of these, administration of intranasal insulin (INI) appears to be the most promising, as it provides a relatively non-invasive, safe, and effective method for bypassing the blood-brain barrier and delivering the ligand directly into the brain [88, 105, 106, 134, 181, 209, 389].

While clinical studies of INI have reported positive impacts on learning and memory [163, 164, 170, 171, 183], it is still unclear which particular pathways mediate these effects. Several potential mechanisms have been suggested, including insulin's impact on cerebral blood flow [179, 390], the ability of insulin to reduce neuroinflammation and oxidative stress [195, 196, 198, 370], insulin-mediated attenuation of age- or AD-related calcium dysregulation [55, 126, 204, 271, 282], and its ability to improve neuronal glucose metabolism [116, 122, 194, 203, 320, 362, 363]. However, although low nanomolar concentrations of insulin are often used to selectively bind the IR, the potential for non-specific activation of other known or unknown receptors (i.e. IGF-I)

still remains. To reduce this potential confound, we recently used a modified, constitutively active form of the human IR (IRβ) [287] to increase insulin signaling through its canonical pathway in the absence of exogenous insulin. While we reported that neuronal IRβ expression maintains elevated insulin signaling in primary hippocampal cultures through activation of the phosphoinositide 3-kinase (PI3K) pathway for at least 48 h, it did not attenuate voltage-gated calcium channel activity in these cells [94]. These results, combined with the complexity of IR signaling in the brain, suggest that other downstream processes should also be considered. In light of this, we focused our attention on neuronal glucose metabolism.

In the periphery, IR signaling triggers activation of the PI3K pathway, which in turn promotes translocation of glucose transporter (GLUT) 4 to the plasma membrane [2, 314, 391, 392] and facilitates the uptake of glucose into muscle and adipose tissue. While the brain and periphery express two distinct isoforms of the IR (IR-A and IR-B, respectively), the overall structures of these receptors are generally comparable. Indeed, while IR-A in the brain has a higher affinity for insulin [13] and is internalized at a much slower rate than the peripheral IR-B [393], evidence shows that both receptors signal through the PI3K pathway and activate many of the same downstream effectors. Thus, it is not unreasonable to assume that IR signaling may also induce GLUT4 activity in the brain. In fact, while the primary GLUT expressed in the brain is GLUT3 [329, 394], recent studies have reported a small, but detectable, amount of the insulin-sensitive GLUT4 in the cerebellum as well as in the hippocampus [387, 395]. As the hippocampus possesses high levels of IR [75, 76, 79], it is possible that insulin acts to increase GLUT4 translocation and glucose uptake in this structure [368], subsequently improving hippocampal processes.

Indeed, recent evidence showed that administration of intracerebroventricular insulin in rats increased GLUT4 translocation to the plasma membrane of hippocampal cells in a PI3K-dependant manner [152], a result that closely mirrors prior reports of increased hippocampal glucose uptake following a spatial learning task in this same animal model [396]. Similarly, other work in cell culture models also reported that acute administration of insulin to hippocampal neurons leads to elevated GLUT4 expression [317].

To test the hypothesis that insulin signaling can stimulate neuronal glucose metabolism, we devised a series of experiments to measure glucose uptake, rates of glucose utilization, and expression of GLUTs 3 and 4 following constitutive IR signaling in mixed, primary hippocampal cultures. We show that this approach increased uptake and indirect measures of utilization of the glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) in neurons, but not astrocytes. These results were corroborated using radiolabeled glucose assays which also indicated elevated glucose uptake in IRβ-expressing cells compared to controls. Additionally, we report an IRβ-associated increase in overall GLUT3 expression, as well as alterations in this transporter's localization within the cell. Surprisingly, no differences in GLUT4 expression or localization were detected. Our results support the hypothesis that insulin signaling is tied to neuronal glucose metabolism in the hippocampus, potentially through the neuron-specific GLUT3. Further, these results provide insight into potential mechanisms mediating the therapeutic benefits of INI administration in the clinic while highlighting the validity of using molecular techniques to study these effects.

6.3 METHODS

6.3.1 Preparation of Mixed, Primary Hippocampal Cultures

Mixed (neuron and glia) primary hippocampal cell cultures were established from Sprague-Dawley rat pups at embryonic day 18 or 19 as described previously [203, 275, 306]. Briefly, hippocampi were first dissected in ice-cold Hank's balanced salt solution (Thermo Fisher Scientific, Waltham, MA) supplemented with 4.2 mM $NaHCO_3$ and 12 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), then transferred to a 50 mL conical tube containing 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and incubated at room temperature (23 °C) for 11 min. Hippocampi were subsequently washed three times with warm (37 °C) Minimum Essential Medium (Thermo Fisher Scientific) supplemented with 200 mM L-glutamine and 35 mM D-glucose (SMEM), then triturated in 10 mL of warm SMEM. Cells were diluted to the desired concentration and plated in 2 mL aliquots onto coated (0.5% poly-L-lysine) 35 mm plastic (Corning, Corning, NY) or glass (Matsunami Glass IND LTD, Osaka, Japan) dishes and incubated at 37 °C, 5% CO_2. Plating densities (200,000-500,000 cells per dish) were later used to normalize tritium (^3H)-glucose uptake values for each experiment. Three days after plating, half of the media in each dish was replaced with 1 mL of a 5-fluoro-2-doxyuridine solution to stop glial cell growth. To return cells to normal glucose oxidation rates and insulin sensitivity levels, all experiments were conducted following a 24 h incubation in a no serum, low glucose (5.5 mM) solution [203]. All data presented were obtained at room temperature between days in vitro (DIV) 14-17.

6.3.2 Lentiviral Construction and Infection of Primary Hippocampal Cultures

Using a lentiviral delivery system, mixed hippocampal cultures received one of two plasmids: a control plasmid containing a neuron-specific synapsin promoter and a fluorescent marker (mCherry), or an experimental plasmid containing the synapsin promoter, mCherry, and the constitutively active IRβ receptor. Both plasmids were constructed from a pHR-SFFV-KRABdCas9-P2A-Cherry backbone vector (gift from Jonathan Weissman, plasmid #60954, Addgene, Watertown, MA) as described previously [94]. Briefly, the synapsin promoter and IRβ sequence were ligated between the AscI and BamHI sites using PCR and standard digestion protocols. The plasmids were converted into lentiviruses by co-transfecting HEK293 cells with the donor plasmid, PsPAX2, and pMD2.G (gifts from Dr. Didier Trono, plasmid #12260 and #12259, Addgene). The viruses were then precipitated into a pellet using 1.4% w/v polyethylene glycol and 50 mM NaCl, resuspended in cold phosphate-buffered saline (PBS), and frozen (-80 °C) until needed.

All dishes were infected on DIV 10 at a multiplicity of infection of 25. Dishes were then immediately returned to the incubator for 48 h to allow ample time for protein expression. Routine confirmation of mCherry fluorescence was performed on both control and IRβ dishes using a spectral analysis camera (Nuance, CRi Inc., Boston, MA). The expression rate of mCherry was ~80% of cells per dish, similar to that reported in our prior IRβ study [94]. Cell number was not directly affected by virus treatment. As expected, plasmid expression appeared to be limited to only neurons, as no detectable fluorescence was noted in astrocytes from IRβ-treated dishes (*data not shown*).

6.3.3 2-NBDG Imaging of Hippocampal Neurons and Astrocytes

To encourage uptake of 2-NBDG, hippocampal cultures were incubated in 3 mL of a HEPES-based imaging solution (10 mM HEPES, 145 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.3) that contained no D-glucose for 15 min at room temperature and air. Following this, dishes received 200 μM 2-NBDG (diluted in sterile ddH_20 and added directly to the glucose-free solution in each dish) and were then incubated for an additional 5 min in darkness (to preserve fluorescence of the glucose analogue). Although dishes were incubated at room temperature and air for a total of 20 min, the cells appeared healthy and morphologically intact. Dishes were subsequently washed in supplemented (10 mM D-glucose) imaging solution 3 times then incubated in 3 mL of supplemented imaging solution at room temp and air on the microscope stage (E600FN; Nikon Inc., Melville, NY) for 3 min. During this time, a field of view (FOV) containing distinct, healthy cells was found (40x immersion objective; 1 FOV per dish). Immediately following the 3 min incubation, 2-NBDG imaging began (exciter centered at 475 ± 40 nm, emitter centered at 535 ± 45 nm, dichroic mirror with a high-pass at ~505 nm; no binning). Sequential images (500 ms exposure) were taken every 30 s for 5 min for a total of 10 images. Phase images of each FOV were also captured and later used to ensure that only morphologically distinct neurons or astrocytes were included in the analysis.

Fluorescent levels (arbitrary gray value) were quantified using Imaging Workbench 5.0 (Indec BioSystems, Santa Clara, CA). Briefly, a hand-drawn region of interest (ROI) was placed around the somatic area of each cell measured using each FOV's phase image as a reference. An ROI was also drawn in an area of the dish devoid of any cellular components in order to obtain the background signal, which was then subtracted from the

2-NBDG fluorescence value of each cell in that dish. 2-NBDG uptake measures were derived from the initial image taken (**Figure 6.1B**). Indirect measures of the rate of 2-NBDG utilization were obtained by calculating the fluorescent signal decay over time (Fig. 6.1B, slope). We report 2-NBDG imaging results from a total of 89 dishes (226 cells, 12 dams). Data are presented as means ± SEM.

6.3.4 Subcellular Fractionation and Western Immunoblots

For Western immunoblots, the cytosolic and total membrane fractions were isolated from hippocampal cultures using a modified subcellular fractionation protocol [26, 356]. Briefly, 8-10 dishes per treatment group were washed with 600 µl of room temperature PBS, lifted in 400-500 µL of a HEPES-based homogenizing buffer (320 mM sucrose, 2 mM EDTA, 2 mM egtazic acid, 20 mM HEPES) that contained protease and phosphatase inhibitors (#P8340 and #P5726, respectively; Sigma-Aldrich, St. Louis, MO), transferred to a sterile 2 mL microcentrifuge tube, homogenized using a Dounce homogenizing pestle (30 strokes), and spun in an ultracentrifuge at 800 x g, 4 °C, for 10 min. The supernatant was removed and transferred to a fresh 1.5 mL tube, and the remaining pellet was then resuspended in 100 µl of homogenizing buffer and spun again at 800 x g, 4 °C, for 10 min. This second supernatant was then added to the first supernatant tube. A portion (~250 µl) of the combined supernatant described above was aliquoted into a separate sterile tube and labeled as the "total membrane fraction." The rest of the supernatant was then spun at 16,000 x g, 4 °C for 30 min. The supernatant from this final spin was removed, placed in a sterile tube, and labeled as the "cytosolic fraction." Samples that were not used immediately were stored at -20 °C. Protein levels were determined using a bicinchoninic acid assay protein quantification kit (Thermo Fisher Scientific) and a microplate reader. To

assess the purity of our subcellular fractions, Western immunoblots for cytosolic or membrane bound protein markers (GAPDH and calnexin, respectively) were performed on fractionated hippocampal cultures. Robust expression of GAPDH along with no detectable calnexin was found in the cytosolic fraction, while the total membrane fraction showed abundant calnexin expression and no detectable GAPDH, indicating that the two cellular compartments were relatively pure (data not shown).

Western immunoblots for GLUT3 and GLUT4 were performed on hippocampal cultures (n = 3 dams) in either duplicate or triplicate within and across gels. Target proteins were assessed using the following: 1° antibodies – GLUT3 #ab41525 1:1000 (Abcam, Cambridge, United Kingdom), and GLUT4 #SC18 1:1000 (gift from Dr. Lawrence Reagan, University of South Carolina); 2° antibody – anti-rabbit HRP-linked IgG #7074S 1:5000 (Cell Signaling Technologies, Danvers, MA). Blots were developed with chemiluminescence and digitally imaged using a G:Box and GeneSys acquisition software (Syngene, Karnataka, India). Mean arbitrary gray values of the target bands were obtained with ImageJ using the gel analysis tool (Version 1.46r; Wayne Rasband, National Institutes of Health, Rockville, MD). To more accurately assess protein levels, target bands of each sample were normalized to the amount of total protein (derived from Ponceau S staining) in their sample lane. Gray values of the control and IRβ target bands were then averaged within groups. To calculate the relative change in protein level, each averaged IRβ gray value was then normalized to the averaged control gray value from the same experiment. Normalized control data are reported as means per experiment, while normalized IRβ data are reported as means ± SEM.

6.3.5 Tritium-Labeled Glucose Uptake Assays

Radiolabeled glucose uptake assays using ^{3}H-glucose were performed on control and IRβ-treated cultures between DIV14-15. Cells were first washed with PBS, then incubated in 1 ml PBS containing 0.1 mM 2-deoxyglucose and 1 mCi/ml 2-deoxy-D (^{3}H) glucose (Perkin Elmer, Boston, MA) for 5 minutes at 37 °C. Cells were again washed with ice-cold PBS and subsequently solubilized in 0.4 mL of 1% sodium dodecyl sulfate for 10 min at room temperature. Cells were counted for 1 min in 4 mL of Biosafe II Complete Counting Cocktail (Research Products International, Mount Prospect, IL) using a Beckman LS6500 scintillation counter (Beckman-Coulter Inc., Brea, CA). Dishes within each individual experiment were combined and averaged. We report ^{3}H-glucose measures derived from a total of 42 dishes (n = 3 dams). Data are reported as group means ± SEM.

6.3.6 Data Filtering and Statistical Analyses

Prior to statistical analysis of 2-NBDG imaging data, any dish with a background slope more than two standard deviations from the mean background slope of its corresponding group was removed from the study. To ensure only cells that took up 2-NBDG at a reliably detectable level were analyzed, both neurons and astrocytes were then further filtered to exclude any cell that did not have a background-subtracted uptake value of 5 or above. After filtering, cells within each dish were averaged, and any dish with a mean gray value more than two standard deviations away from the total group mean were deemed outliers and removed from the analysis. We report on 2-NBDG uptake measures of 169 neurons (97 control and 72 IRβ) from 67 dishes (control n = 37, IRβ n = 30) and 57 astrocytes (31 control and 26 IRβ) from 22 dishes (control n = 11, IRβ n = 11).

For measures of 2-NBDG utilization rates, hippocampal neurons received an additional filter to exclude any neuron that did not have a negative utilization rate (slope) of -0.1 or less, as this was calculated to be more than 2 times steeper than the average background slope of either group (mCherry background slope = 0.1, IRβ background slope = 0.04). This neuronal utilization filter (defined here as the bleaching correction) was used to ensure that the reported signal decay was due to biological processes rather than a bleaching effect (i.e., the tendency of a glass-bottom dish to become darker over the course of imaging). Astrocytes did not receive a bleaching correction, as they presented with relatively flat or slightly positive slopes in our study. As with measures of uptake, cells within each dish were averaged and statistical outliers were removed from the analysis. We report on 2-NBDG utilization rates derived from 102 neurons (53 control and 49 IRβ) from 38 dishes (control n = 18, IRβ n = 20) and 57 astrocytes (31 control and 26 IRβ) from 21 dishes (control n = 11, IRβ n = 10). Virus effects on 2-NBDG imaging endpoint measures were calculated using student's T-tests (unpaired, 2-tailed, Welch's correction for unequal variances).

For ^{3}H-glucose measures, any dish that was two standard deviations away from the total group mean was deemed an outlier and removed from the analysis. Virus effects on ^{3}H-glucose uptake were determined using a student's t-Test (unpaired, 2-tailed, equal variance). For GLUT3 and GLUT4 Western immunoblots, virus and subcellular fraction effects on endpoint measures were determined using 2-way ANOVAs (repeated measure, Bonferroni post hoc tests). Significance for all comparisons in this study was set at $p < 0.05$.

6.4 RESULTS

6.4.1 2-NBDG Fluorescent Imaging of Primary Hippocampal Cultures

To test if chronic, sustained elevations in IR signaling could influence glucose metabolism, we used 2-NBDG, a glucose analogue that indirectly reports on rates of glucose utilization through the loss of its fluorescence over time [203, 397]. Compared to our previously published rates of glycolysis in hippocampal neurons and astrocytes [203], the uptake and utilization rates measured here were reduced, likely because these experiments were conducted at room temperature. Nevertheless, it is doubtful this would alter measures in one cell-type compared to another. 2-NBDG was successfully taken up by both neurons and astrocytes. Analysis of initial 2-NBDG images revealed that uptake was significantly elevated in IRβ-expressing neurons compared to controls ($p = 0.019$; Fig. 6.1B and 6.1C), with some IRβ dishes having more than twice the amount of 2-NBDG signal (Fig. 6.1B). Similarly, IRβ expression was associated with significantly faster rates of 2-NBDG utilization ($p = 0.013$), as indicated by a significantly steeper slope of signal decay in these same cells compared to controls (-2.066 vs. -0.548, respectively; Fig. 6.1B and 6.1D). Much like measures of 2-NBDG uptake, many of these slopes were more than two-times higher than the average control neuron (Fig. 6.1B). Visual observation of the cells during imaging mirrored our statistical analysis, with neurons from IRβ dishes showing robust and easily distinguishable fluorescent signal compared to the more subdued signal from the control dishes (Fig. 6.1A).

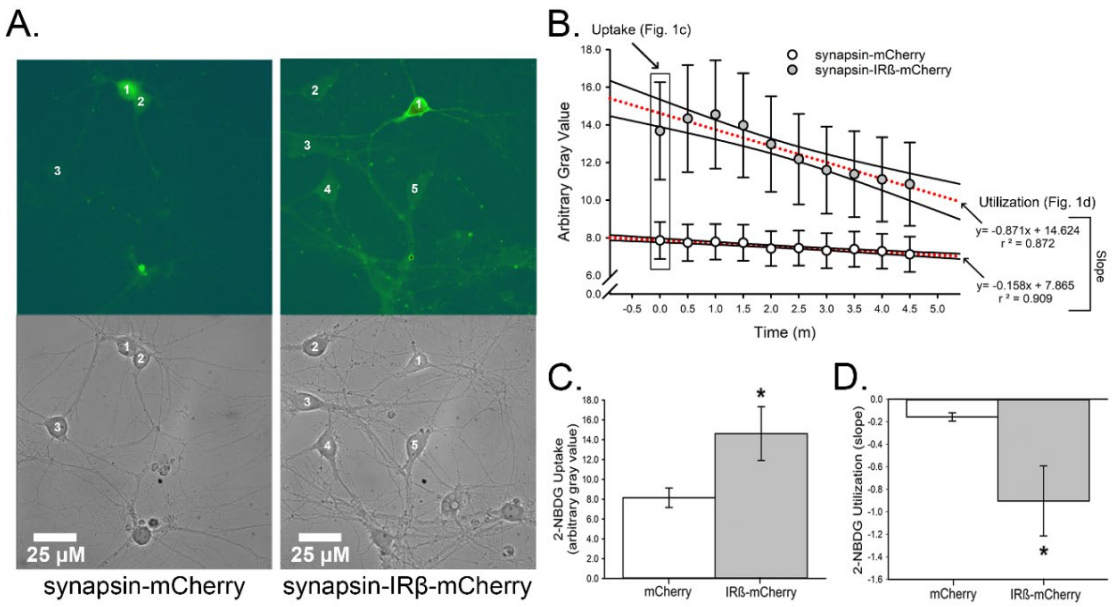


Figure 6.1 2-NBDG imaging of primary hippocampal neurons with or without expression of IRβ. (A) Representative phase and 2-NBDG fluorescent photomicrographs obtained from hippocampal cultures. Numbers 1-4 indicate distinct neurons. **(B)** Data obtained from a representative control and IRβ neuron following 2-NBDG imaging. Arbitrary units were derived from mean gray values. Boxed data at time point 0.0 indicate initial gray values used for 2-NBDG uptake analysis. Red lines indicate linear regressions used for Δ2-NBDG calculation. **(C)** Quantification of background-subtracted 2-NBDG uptake in hippocampal neurons with or without IRβ expression. Significant elevation in 2-NBDG uptake was observed in IRβ-expressing neurons (n = 30) compared to controls (n = 37) (student's T-test, p = 0.02). **(D)** Quantification of background-subtracted Δ2-NBDG used to infer indirect rates of glucose utilization. A significant increase in the rate of 2-NBDG utilization was observed in IRβ-expressing neurons (n = 20) compared to controls (n = 18) (student's T-test, p = 0.01). All data represent means ± SEM. Asterisks (*) indicate significance at $p < 0.05$.

Imaging of astrocytes showed no statistically significant difference in 2-NBDG uptake in control dishes compared to dishes receiving the IRβ plasmid ($p > 0.05$; **Figure 6.2B and 6.2C**). Similarly, utilization rates did not differ between the two groups ($p > 0.05$), and slope averages were relatively flat (control: 0.068, IRβ: 0.035). Visual observation showed a much lower level of fluorescent signal in astrocytes (Fig. 6.2A) compared to neurons (Fig. 6.1A). These results provide evidence for neuronal-selectivity of the synapsin promoter in our lentiviral constructs. Additionally, the low level of astrocytic 2-NBDG uptake may reflect their use of alternative energy sources, such as glycogen [398, 399].

6.4.2 ^{3}H-Glucose Uptake in Primary Hippocampal Cultures

To further corroborate IRβ's ability to elevate glucose metabolism, we performed an additional analysis using a radiolabeled glucose uptake assay. Scintillation counts reflective of ^{3}H-glucose uptake highlighted a significant increase in IRβ-expressing cells (student's t-Test; $p = 0.04$), which exhibited ~30% more uptake than controls (**Figure 6.3A**). To control for cell density in these measures, we compared protein quantifications (Ponceau S staining of Western immunoblots; $n = 3$ dams, 25 dishes per dam) between control and IRβ dishes. A small, nonsignificant elevation in the number of control cells was seen compared to those expressing IRβ (*data not shown*). Even a small elevation in cell number in control dishes would underestimate the significance of greater ^{3}H-glucose uptake in the IRβ dishes we report here.

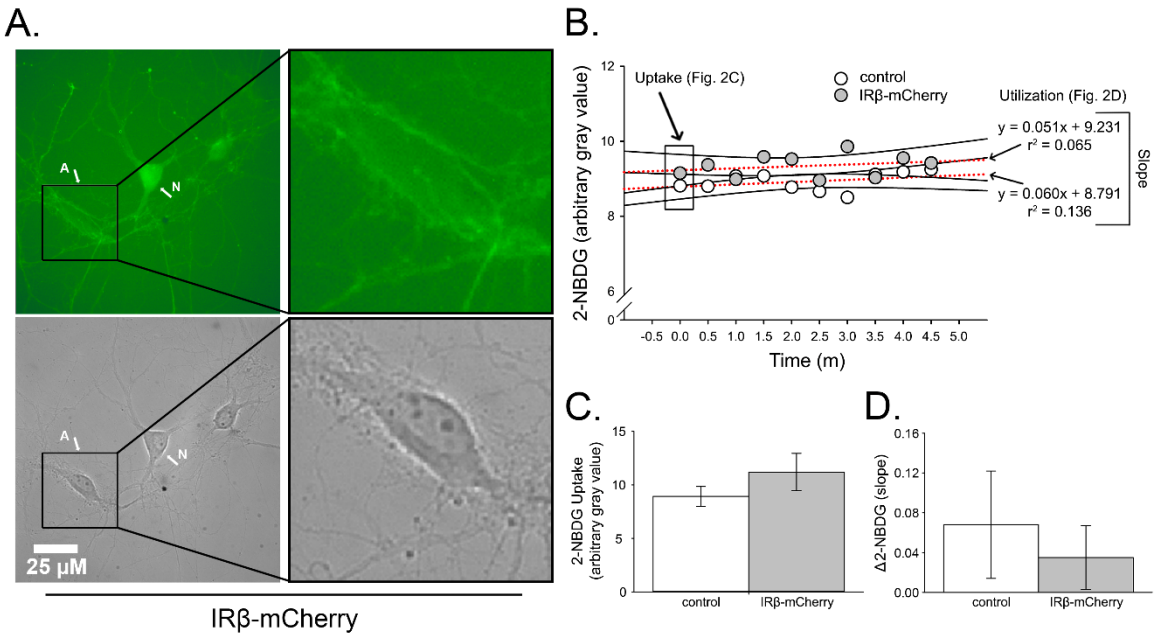


Figure 6.2 2-NBDG imaging of primary hippocampal astrocytes from dishes with or without IRβ-expressing neurons. (A) Representative 2-NBDG fluorescent (top) and phase (bottom) photomicrographs obtained from a single IRβ-expressing hippocampal culture dish. The letter A indicates a distinct astrocyte. The letter N indicates a distinct neuron. Right panels provide greater detail of astrocyte morphology and highlight the visual reduction in 2-NBDG fluorescent signal in this cell compared to the neighboring neuron. **(B)** Data compiled from control (n = 11) and IRβ (n = 11) groups following 2-NBDG imaging. Arbitrary units were derived from mean grey values. Boxed data at time point 0.0 indicate initial gray value used for 2-NBDG uptake analysis. Red lines represent examples of the linear regressions used for Δ2-NBDG calculation. **(C)** Quantification of background-subtracted 2-NBDG uptake in hippocampal astrocytes from dishes with (n = 10) or without (n = 11) IRβ expression. No significant changes in 2-NBDG uptake values were observed between control and IRβ dishes (student's T-test, $p > 0.05$). **(D)** Quantification of background-subtracted Δ2-NBDG as indirect measures of 2-NBDG

utilization rates in astrocytes. No significant difference in rates of 2-NBDG utilization were noted between control and IRβ dishes (student's T-test, $p > 0.05$). All data represent means ± SEM.

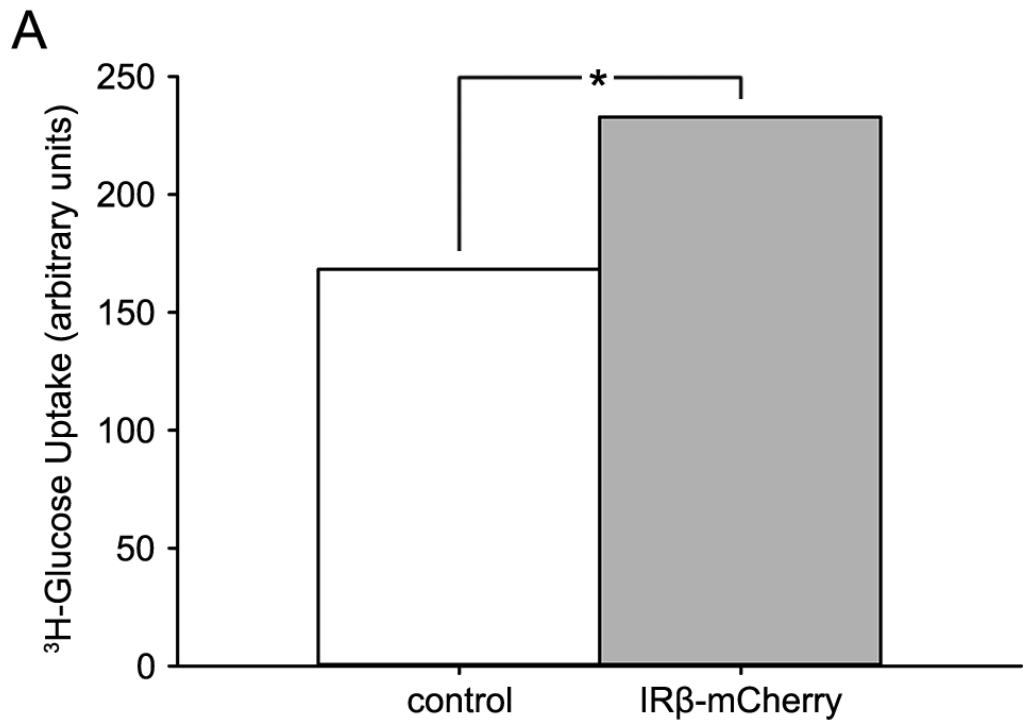


Figure 6.3 Quantitative analysis of ^{3}H-glucose uptake in primary hippocampal cultures. (A) Relative levels of ^{3}H-glucose uptake in mixed, primary hippocampal cultures with or without IRβ expression. Data were derived from a total of 3 separate preps, each from a different dam (n = 3). A significant elevation in radiolabeled glucose uptake was observed in the IRβ-expressing dishes compared to control dishes (student's T-test, $p = 0.04$). All data represent means ± SEM. Asterisks (*) indicate significance at $p < 0.05$.

6.4.3 Western Immunoblots of GLUT3 and GLUT4

To test if elevations in glucose uptake and utilization rates were due to increased GLUT3 or GLUT4 expression, we performed Western immunoblot analysis across cytosolic and total membrane subcellular fractions. Results of GLUT3 immunoblots indicated a significant overall effect of IRβ (2-way ANOVA; $F_{(1,8)} = 5.84$, $p = 0.04$) (2-way ANOVA, $F_{(1,8)} = 5.84$, $p = 0.04$; **Figure 6.4A and B**), with IRβ-expressing cells showing elevated levels of GLUT3 compared to controls. This was particularly notable in the total membrane fraction, where IRβ correlated with a ~30% elevation in GLUT3 expression (Bonferroni post hoc; $p < 0.05$). Surprisingly, no significant differences in GLUT4 expression were detected between control and IRβ-expressing cells in either subcellular fraction (2-way ANOVA; $p > 0.05$) (2-way ANOVA, $p > 0.05$; Fig. 6.4C and D).

6.5 Discussion

The current study was conducted to test the hypothesis that sustained IR signaling could mediate aspects of glucose metabolism and/or alter expression of GLUT3 and GLUT4 in hippocampal cultures. We show that constitutive IR activation conferred from IRβ expression is able to significantly increase both glucose uptake and utilization rates, as well as upregulate the total membrane expression of the neuron-specific GLUT3. These results suggest that insulin signaling in the brain may target pathways associated with GLUT translocation, particularly in areas of the brain associated with memory and learning. Further, while not a direct measure of IRβ's impact on neuronal survival, we do see here that sustained activity of the IR does not negatively interfere with cell health.

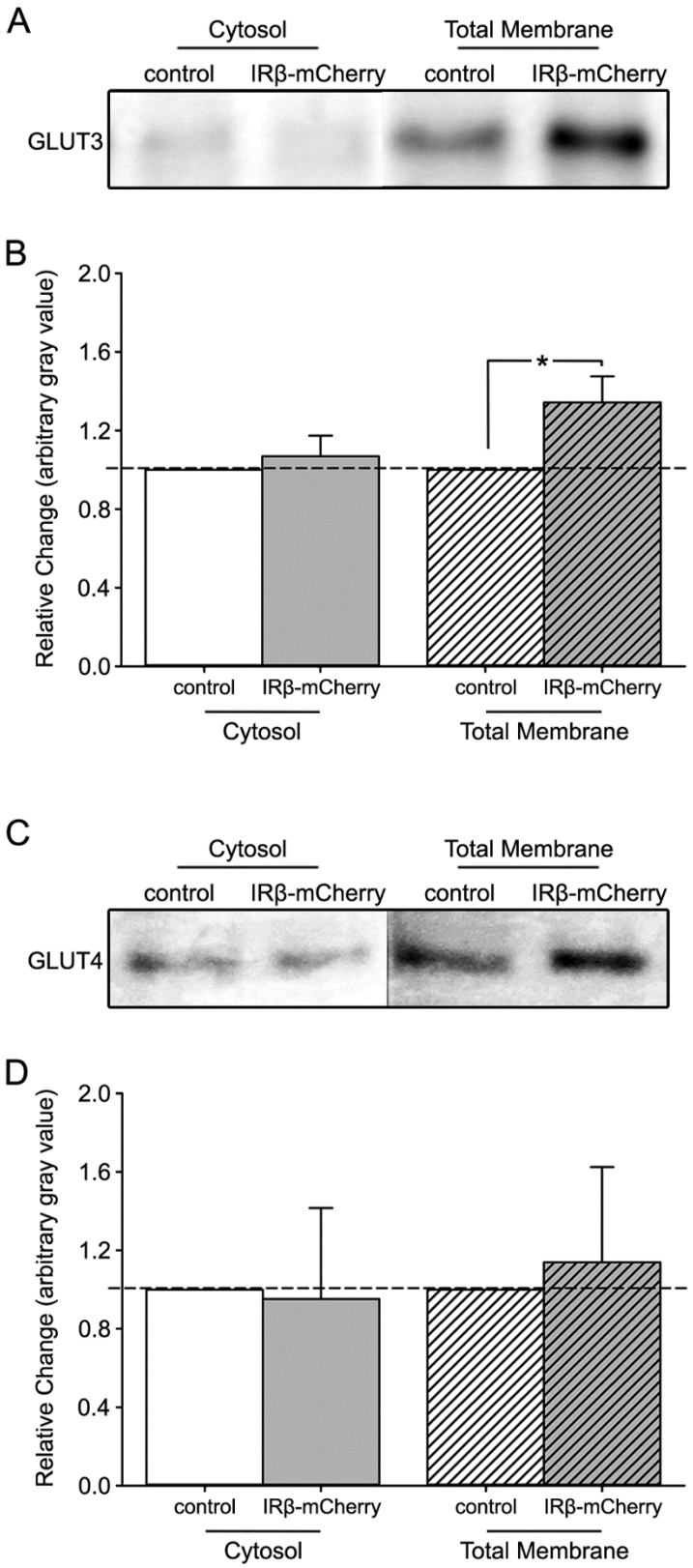

A
Cytosol
Total Membrane
control
IRβ-mCherry
control
IRβ-mCherry
GLUT3
B
Relative Change (arbitrary gray value)
2.0
1.6
1.2
0.8
0.4
0.0
*
control
IRβ-mCherry
control
IRβ-mCherry
Cytosol
Total Membrane
C
Cytosol
Total Membrane
control
IRβ-mCherry
control
IRβ-mCherry
GLUT4
D
Relative Change (arbitrary gray value)
2.0
1.6
1.2
0.8
0.4
0.0
control
IRβ-mCherry
control
IRβ-mCherry
Cytosol
Total Membrane

Figure 6.4 Western blot analysis of fractionated hippocampal cultures with or without expression of IRβ. (A) Representative Western immunoblots of cellular fractions derived from primary hippocampal cultures (cytosol fraction, left; total membrane fraction, right) probed for GLUT3. **(B)** Quantification of the relative change in GLUT3 expression between cytosol and total membrane fractions of hippocampal cells. A significant overall effect of virus was detected (2-way ANOVA; $F_{(1,8)} = 5.88$, $p = 0.04$). Bonferroni post hoc tests revealed that IRβ-expressing cells have significantly higher levels of GLUT3 in the total membrane fraction compared to control cells ($p < 0.05$). A trend between the cytosol and total membrane fractions (2-way ANOVA; $F_{(1,8)} = 2.61$, $p = 0.15$) and an interaction trend indicating a differential effect of virus between these two fractions (2-way ANOVA; $F_{(1,8)} = 2.61$, $p = 0.15$) were also noted. **(C)** Representative Western immunoblots of cellular fractions probed for GLUT4. **(D)** Quantification of the relative change in GLUT4 expression between cytosol and total membrane fractions of hippocampal cells. No effects of virus (2-way ANOVA; $F_{(1,8)} = 0.02$, $p > 0.05$), cellular fraction (2-way ANOVA; $F_{(1,8)} = 0.08$, $p > 0.05$), or the interaction (2-way ANOVA; $F_{(1,8)} = 0.08$, $p > 0.05$) on GLUT4 expression were detected between control and IRβ-expressing cells. All control data represent means. All IRβ data represent means ± SEM. Asterisks (*) indicate significance at $p < 0.05$.

6.5.1 Insulin Signaling May Mediate Glucose Metabolism in the Brain

Data derived from IRβ-expressing neurons showed that constitutive IR activation was associated with increased uptake of the glucose analogue 2-NBDG as well as ^{3}H-glucose compared to controls (Fig. 6.1B and 1C, Fig. 6.3). In the periphery, insulin is a key regulator of glucose uptake in adipose and muscle tissue; therefore, it is not surprising that similar results are found in the CNS. Indeed, prior work using 2-(18F)fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) imaging to study brain glucose metabolism in animal models has shown that INI administration can increase glucose uptake following traumatic brain injury [194] and streptozotocin (STZ)-induced Type-2 diabetes mellitus [116], two phenotypes known to present with varying degrees of CNS hypometabolism in the clinic [38, 378, 379, 381, 382, 386, 400]. Another FDG-PET study, this time in humans, reported that intravenous insulin infusion following somatostatin-induced inhibition of basal insulin secretion was able to significantly elevate cerebral glucose uptake in healthy male subjects [401]. Reductions or alterations of glucose uptake and other markers of glucose metabolism have also been identified in AD patients [139, 211, 370, 380, 401, 402], with some studies suggesting that these perturbations may begin long before the onset of clinical AD symptoms Similarly, in cognitively-normal aged individuals, glucose metabolism appears to be markedly reduced in multiple brain areas [383]. Clearly, these results provide a strong rationale for the use of INI or other therapeutic strategies to increase IR signaling in the brain, and also support further investigations in other animal models of neurodegeneration.

Along with elevations in uptake, we also provide evidence of increased 2-NBDG utilization rates in IRβ-expressing neurons compared to controls (Fig. 6.1B and D). As the

rate of glycolysis is directly dependent on the amount of intracellular free glucose and/or previously phosphorylated glucose (glucose-6-phosphate), it is unsurprising that elevations in both measures were detected simultaneously within the same cells. Unlike neurons, astrocytes from IRβ-infected dishes did not show an increase in 2-NBDG metabolism, despite elevated 2-NBDG uptake and utilization occurring in IRβ-expressing neurons (Fig. 6.2B, C, and D). It has been suggested that astrocytes are metabolically active and may also supply neighboring neurons with lactate produced during anaerobic glucose metabolism (i.e. the astrocyte-neuron lactate shuttle) [327, 403]. However, more recent reports have proposed that neurons are capable of independently, and perhaps even preferentially, converting glucose into lactate themselves [203, 404, 405]. If neurons primarily relied on lactate supplied by astrocytes, one would expect to see a parallel increase in astrocytic glucose uptake and utilization in response to increased metabolism in IRβ-expressing neurons. Thus, the results presented here support an alternative hypothesis: that neurons are capable of metabolizing glucose directly.

6.5.2 IR Signaling May Regulate GLUT3 Expression in the Hippocampus

Results from Western immunoblots highlighted an overall effect of IRβ on GLUT3 expression, with a significant elevation noted in the total membrane fraction (Fig. 6.4A and B). GLUT3 is the primary neuronal GLUT and is distributed within numerous areas of both the human and rodent brain, particularly the cerebral cortex, cerebellum, and hippocampus [342-344, 347]. The robust expression of GLUT3 in the hippocampus, along with evidence of reduced spatial memory in GLUT3-deficient mice [406] and the high subcellular localization of GLUT3 to synaptically-dense areas such as the neuropil and neuronal processes [348-351] suggests this transporter may serve a vital role in learning,

memory, and synaptic transmission. GLUT3's regulation has been thought to depend on specific triggers such as hypoxia and glucose depravation [332, 338, 352, 353, 407], oxidative stress and fatty-acid availability [354, 357], brain development and aging [359, 365, 408], and neuronal activation and synaptic transmission [358]. Thus, alterations in overall cellular metabolism might indirectly impact GLUT3 expression or translocation. However, recent evidence has also suggested that insulin and insulin-related processes may directly influence this transporter. An early study using cell fractionation techniques in muscle cells showed that both insulin and IGF-I stimulated a significant elevation in translocation of GLUT3 from the cytosol to the plasma membrane [360]. Others have shown that excess thyroid hormones can increase insulin-stimulated GLUT3 recruitment to the plasma membrane of monocytes [355]. Furthermore, recent evidence suggests that insulin-mediated modulation of GLUT3 may also occur in the brain. In fact, a study in primary hippocampal cultures indicated that in vitro administration of insulin could significantly increase translocation of GLUT3 vesicles to the plasma membrane, although fusion of the vesicles and the elevations in neuronal glucose uptake required a KCl membrane depolarization following initial treatment with the ligand [363].

With respect to the insulin-sensitive GLUT4, we did not see changes in the overall expression level or subcellular localization of this transporter following IRβ expression (Fig. 6.4C and D). However, others have shown that insulin is indeed capable of modulating GLUT4 in the brain through the canonical IR signaling pathway (i.e. PI3K) [152], and that this process plays an important role in hippocampally-mediated spatial memory [115, 152, 317]. Compared to other studies that used acute insulin administration, we used a chronic activation of the IR signaling pathway, which could explain the lack of

effect on GLUT4 presented here. In fact, the brain IR isoform does not appear to be downregulated following sustained activation [22, 86]; therefore, constitutive IRβ activity may have triggered a compensatory mechanism that prevented elevation of GLUT4 at the plasma membrane. This theory is supported by recent work from our lab showing that long-term, chronic INI administration (3 months) of insulin aspart did not alter spatial learning and memory on the Morris water maze task in either young or aged Fisher 344 rats [95], whereas more acute, shorter-term exposures (8-11 days) using INI detemir and lispro significantly improved behavioral performance in this same animal model [126]. Similarly, another study reported that long-term (30-60 days), repeated INI in mice does not significantly improve olfactory object-recognition memory compared to more acute exposures to the ligand [192]. Clearly, additional work investigating the particular pathways and mechanisms involved with insulin's regulation of brain energy metabolism and its relationship to GLUTs is needed.

The work presented here demonstrates that: 1.) expression of a modified, constitutively active human IR (IRβ) significantly elevates 2-NBDG uptake and rates of utilization in cultured hippocampal neurons; 2.) astrocytes may be less metabolically active compared to neighboring neurons within the network; and 3.) chronic IR signaling in hippocampal cultures is associated with increased GLUT3 expression, particularly in the total membrane subcellular fraction. Our results not only support the increasing evidence that IR signaling plays a vital role in brain metabolism by regulating glucose uptake and usage, but also suggests a potential mechanism (i.e. GLUT3) behind INI's beneficial effect on memory and learning in the clinic. Additionally, the molecular techniques used here

highlight a new approach to study chronic IR signaling without the need for exogenous ligand delivery.

6.6 ACKNOWLEDGEMENTS

The authors acknowledge the use of facilities in the University of Kentucky Center for Molecular Medicine Genetic Technologies Core. This core is supported in part by the National Institutes of Health (P30GM110787).

6.7 FUNDING

This work was supported by the National Institutes of Health [R01AG033649, T32DK007778, T32AG057461]; the University of Kentucky College of Medicine, Lexington, KY [Fellowship to HF]; and the University of Kentucky Department of Pharmacology and Nutritional Sciences [Reinvestment Fund Award].

CHAPTER 7. DISCUSSION AND FUTURE DIRECTIONS

The studies I completed as part of this dissertation were designed to investigate the cognitive effects and cellular mechanisms involved with IR signaling in the brain. Over the past 3 decades, the field of neuroscience has made considerable strides regarding this topic, yet it is increasingly clear that insulin's modulation of physiology in the brain cannot be explained through a single pathway or even a small series of events; instead, it appears that IR signaling is capable of regulating a wide range of processes, including, but certainly not limited to, calcium homeostasis, neuroinflammation, CNS metabolism, cerebral blood flow, and synaptic plasticity. The manuscripts I have presented here, as well as my review of over 30 years of literature, demonstrate the dynamic interplay of cellular mechanisms mediated by IR signaling well, and introduce novel findings regarding insulin's relationship to gene expression and glucose metabolism in the hippocampus.

7.1 Duration, Dose, and Formulation: Potential Factors Mediating INI Efficacy

The use of INI to improve learning and memory is rapidly gaining traction as a beneficial therapeutic in the clinic. Like many others, we have investigated this technique in the past and reported positive results on the improvement of spatial learning and memory recall in F344 rats; yet, the INI study presented in this dissertation (see Chapter 2) does not align well with our prior studies, as neither young nor aged F344 rats receiving INI showed a statistically significant improvement on the MWM spatial memory test compared to controls. This was somewhat unexpected for us, as our investigations of two other insulin formulations (insulin lispro and detemir) using similar techniques, doses, and animals

substantially improved performance on these same measures [126]. However, during another recent study, we also did not detect a substantial improvement in behavioral performance in either young or aged F344 rats following INI administration of insulin glulisine [127], raising the question as to whether insulin formulation or treatment duration can perhaps influence the efficacy of this therapy.

7.1.1 Fast-Acting Insulin Analogues

Prior studies investigating INI in both humans and animal models have used a variety of different insulin formulations, including endogenous forms of the ligand (regular human insulin) as well as synthetic insulin analogues, such as insulin lispro, detemir, glulisine, and aspart. These synthetic formulations all exert overall effects in the periphery that are similar to those of the endogenous ligand, but they differ slightly from regular human insulin in terms of their chemical structure and kinetics [230]. The modifications added to synthetic insulin analogues were initially included to improve the drugs' absorption rate and time course of action (fast-acting vs. slow-acting); however, the impact of these structural and kinetic changes on CNS physiology are still unclear.

Of the 4 insulin analogues we have tested using INI in our lab, 3 have been "fast-acting." These 3 analogues (insulin lispro, glulisine, and aspart) differ from regular human insulin by 1 amino acid. This alteration reduces the likelihood that the insulin monomers will form hexameric complexes, thus speeding up the their absorption in the periphery [409]. In addition to this amino acid substitution, the synthetic analogue insulin glulisine also lacks zinc, the central ion involved with insulin hexamer formation. INI glulisine, while potentially a beneficial therapy with respect to aspects of memory and learning [164, 173], has also led to mild detrimental side-effects in the clinic, including rhinitis and nose-

bleeds. These side-effects eventually lead to temporary suspension of the SNIFF Trial, a clinical study using this particular formulation [171], and are likely caused by the addition of stabilizing compounds (e.g. cresol and phenol) included to improve the shelf-life of the drug. In our work, insulin glulisine did not appear to detrimentally affect animals receiving the treatment, but the significant decrease in behavioral improvements between glulisine and other formulations (i.e. insulin lispro and detemir) imply that the chemical structure of different insulin analogues may influence their ability to ameliorate cognitive decline when administered intranasally.

Due to the lack of a clear behavioral improvement following INI glulisine administration, we then chose to look at another insulin formulation: aspart. Insulin aspart, like glulisine, is considered a fast-acting analogue. This particular formulation not only significantly increases both the maximum level and rate of glucose infusion compared to regular human insulin but can also be absorbed up to twice as fast [231]. In addition to its improved peripheral kinetics, INI administration of aspart has also been shown to enhance declarative memory more so than INI using regular insulin [207], suggesting that its rate of absorption through the nasal mucosa may also be elevated. While the justification for using insulin aspart in our most recent animal INI study was strong, we again detected no observable, statistically significant drug effects on hippocampal spatial learning and memory performance [95]. As stated previously, these results were discouraging; if the insulin formulation is not the explanation for these contradictory results, what other aspects of INI treatment could potentially mediate these differences? It was then that we began to consider the impact of treatment duration.

7.1.2 Impact of Treatment Duration on INI Efficacy

When discussing the effect of time on our outcome measures, we must first place our INI studies into one of 2 categories: studies of *acute* INI treatment or studies of *chronic* INI treatment. With respect to our prior work, acute INI treatment refers to dosing regimens that would either induce repeated, fluctuating elevations in brain IR signaling over a brief period of time (e.g. 1 dose of INI lispro or detemir per day for 8-11 days) or induce a single, short-lived elevation immediately prior to behavioral testing (e.g. 1 dose of INI lispro or detemir 1-3 h prior to behavioral testing) [126]. Chronic treatment, on the other hand, refers to the repeated, consecutive administration of INI over many months, thus leading to sustained, high levels of IR signaling over a long time-period (1 dose of INI aspart per day, 5 days a week, for 12 weeks) [95].

When comparing our studies, it is intriguing to note that those using acute, transient INI lispro or INI detemir resulted in improved memory and learning, whereas our study of chronic, long-term INI aspart did not show these same effects in spite of increasing IR signaling and altering gene expression. However, we are not the only investigators to report such findings. A study performed in adult C57BL6/J mice showed that unlike acute INI, long-term INI administration (twice per day for 30 or 60 days) did not enhance olfactory odorant discrimination, reversal learning, or object memory recognition, though it did significantly increase pIR levels [192]. These investigators proposed that long-term insulin may either induce brain insulin resistance or dampen behavioral effects by increasing CNS insulin concentrations to levels above those that are beneficial. Additionally, another group recently provided evidence of IR-mediated GLUT4 downregulation following sustained

receptor activity in adipose tissue [410], further supporting the theory that chronic INI or IR activation is functionally distinct from more acute, transient insulin events.

Perhaps, in situations of chronic IR signaling, such as those induced by repeated, long-term INI administration, inhibitory pathways are triggered and subsequently activate compensatory mechanisms that attenuate IR-mediated processes. This may be especially true for IR-A in the brain, as this isoform appears to be internalized at a much slower rate and possesses a higher binding affinity for insulin than the peripheral IR [12, 13, 86]; in turn, these characteristics could produce significantly higher levels and longer durations of IR signaling compared to that of IR-B in the periphery. However, it is important to clarify that if these compensatory mechanisms do exist, they do not seem to impact all insulin-related processes equally, as INI aspart was still able to significantly elevate IR signaling and alter gene expression in the hippocampus [95].

7.2 Challenging the Theory of Insulin Resistance in the Aged Brain

As stated in Chapter 1, section 1.3.4, the discovery of reduced brain IR density and signaling markers led many researchers to suggest that cognitive decline associated with aging and AD may be a result of receptor desensitization, much like that seen in the periphery of T2DM patients. However, the work presented in my first manuscript (Chapter 2) indicates that this may be an overly simplistic view of IR activity in the aged and AD brain. Indeed, while our study of aged animals receiving INI aspart did not appear to differ from aged controls on measures of spatial learning and memory, they did have significant alterations in their gene transcriptome [95]. Additionally, our previous work using other

formulations of INI in this animal model also showed changes in CBF, elevations in IR signaling markers, and improved spatial behavior in aged animals [126, 127].

In fact, one of our studies in particular actually reported that hippocampal brain slices from aged animals responded with a greater decrease in the calcium-dependent AHP than slices from younger animals [126]. These results, in combination with numerous studies of INI in the clinic and in animal models that also highlight cognitive improvements in older individuals or aged animals, imply that the aged brain is *not* unresponsive to insulin, and may, in fact, even be *more sensitive* than the young brain. While seemingly contradictory to early reports of reduced IR signaling and density in aged and AD brains [129], this theory of elevated receptor sensitivity during aging could be explained as the aged brain's attempt to compensate for reduced insulin transport into the CNS by either overexpressing IR at the plasma membrane or by conferring modifications to IR structure that may allow it to function at a more efficient level. Still, it remains to be determined whether or not the aged and/or AD brain truly experiences the classical form of insulin resistance seen in the periphery. Future investigations regarding this particular topic are needed, as the level of brain IR sensitivity in these elderly patients could greatly impact the type of treatments needed to ameliorate these pathologies in the clinic.

7.3 Implications for the Use of Molecular Techniques to Elevate IR Signaling

In addition to providing novel evidence of insulin's effect on hippocampal glucose metabolism and gene expression, the work completed during my dissertation also presented the successful implementation of a molecular approach for elevating IR signaling in the

absence of the ligand. This technique involved the use of a modified, constitutively active human IR that conferred sustained downstream signaling but lacked a functional insulin binding site [287]. This construct was created by truncating roughly 2680 bp from the α-subunit of the endogenous human IR-A isoform expressed, resulting in a 1430 bp receptor that we called IRβ. This receptor is substantially smaller than the normal human IR: ~50 kDa compared to ~150 kDa. Additionally, IRβ does not appear to be capable of internalizing for receptor degradation/recycling like the endogenous IR [287].

The sustained signaling and lack of downregulation initial raised concerns that the receptor may negatively impact neuronal health in our cell culture experiments; surprisingly, however, we have yet to detect a noticeable difference in cell density, survival rate, morphology, or growth in our IRβ-expressing cells compared to controls. Additionally, shortly after completion of my final dissertation project (see Chapter 6), we moved forward with our goal of studying this receptor *in vivo* and constructed a similar IRβ-containing adeno-associated virus (AAV) for stereotaxic injection into young and aged F344 rats. Much like our studies in culture, we have yet to observe any detrimental effects of the receptor on neurological health, food intake, weight, behavior, or any other easily measurable physical attributes (*unpublished data*).

These most recent results are exciting, as they support our initial hypothesis that employing molecular methods to elevate signaling in the absence of insulin could be a useful way to study the direct effects of IR activity in the hippocampus without negatively impacting cell and/or whole-body health. To our knowledge, use of a purely molecular technique in this manner had yet to be explored prior to our investigations. Thus, we feel that demonstrating the effectiveness of this method will allow other researchers to consider

alternative options that may help reduce variability and confounding factors associated with delivering the ligand to cell culture or animals.

7.4 Study Limitations

Regardless of the stringency employed when designing experimental studies, there will always be limitations that arise, and the manuscripts I completed during my dissertation are no exceptions. The most prominent of these would undoubtably be the impact of time during both hippocampal cell culture studies (see Chapters 4 and 6). The use of lentivirus to express a protein of interest is a common molecular technique. While relatively safe, use of these viruses is still considered to be a BSL2-level laboratory protocol which requires specialized lab space and equipment. One safety measure in particular is the inability to remove cells that have come into contact with said virus for 48 h after initial infection. Unfortunately, due to the physical distance between our BSL2 certified lab space and other equipment used during my studies, such as microscopes, bench space reserved for protein assays, and shared centrifuges, this regulation meant that we were only able to measure the cellular effects of IRβ expression 2 days after viral treatment of the cells. As the IRβ receptor is constitutively active, this resulted in our hippocampal cells undergoing sustained IR signaling for at least 48 h prior to recordings or observations being undertaken. Unfortunately, this does introduce the potential confound of time.

Sustained IR signaling, while not necessarily neurotoxic, may lead to a variety of yet unknown cellular effects that could influence the results obtained during our work. Interestingly, a recent study actually indicated that prolonged IR signaling lead to an increase in oxidative stress which in turn downregulated the expression of the insulin-

sensitive GLUT4 in adipocytes [410]. The canonical IR signaling pathway in the periphery involves insulin-mediated upregulation of GLUT4 to the plasma membrane following receptor activation and downstream signaling (see Chapter 1, section 1.2.2). The observation that sustained IR signaling *downregulates* GLUT4 is intriguing, as it implies that the duration of IR signaling may drastically alter the pathways that subsequently are activated. If this is true, the effects measured as part of my studies may be different than detected after acute or transient exogenous insulin administration. In fact, this could also potentially explain the discrepancies between my results on insulin's impact on VGCC currents (see Chapter 4) and those reported by a previous graduate student using exogenous insulin application to either hippocampal brain slices or to the same cell culture system used in my own work [126, 204].

7.5 Future Directions

7.5.1 Astrocyte-Specific IR Signaling

While the existence of a direct connection between IR signaling and metabolism in the brain seems likely, the characterization of this relationship is still in its infancy. With respect to techniques similar to those used during my dissertation project, a number of new avenues could be explored that would help expand our knowledge regarding the impact of insulin on hippocampal glucose uptake and utilization.

One particular topic that needs more focus is the involvement of hippocampal astrocytes in neuronal energy processes. It is only relatively recently that we discovered neurons were capable of independently metabolizing glucose into usable energy [404]. In fact, the prevailing theory was actually that astrocytes were the primary drivers of glucose

metabolism in the brain, converting nearly all available free glucose into CO_2 before switching to anaerobic glycolysis and lactate production under heavy cognitive demand. At this point, the astrocytes would then transfer this lactate to nearby neurons in a process known as the "astrocyte-neuron lactate shuttle" [327, 403].

Although this theory has now been contested, it does not necessarily preclude astrocytes from being key players in CNS metabolism. Indeed, the reported presence of the IR-B isoform in these cells [80, 81] further implies that they may possess metabolic pathways separate from those used by neurons, and that these pathways may respond to insulin in functionally distinct ways. Results derived from my study of constitutive IR activity and glucose metabolism in primary hippocampal neurons did not indicate that astrocytes were responding to the elevated metabolism that was occurring in neighboring IRβ neurons; however, we cannot discount the possibility that astrocytes are mediating neuronal physiology in ways other than the direct uptake or utilization of free glucose.

Additionally, it is important to note that our lentiviral constructs contained synapsin, a neuron-specific promoter that inhibits expression of the IRβ receptor in astrocytes or glial cells. Perhaps my results would have been different had we also expressed this constitutive IR in both cell-types. The use of the GFAP promoter to confer astrocyte-specific expression of plasmids is a commonly employed technique in molecular labs, and we have also had significant experience utilizing this method in similar cell-types and under comparable conditions. Therefore, expressing IRβ in astrocytes only may be a useful and relatively simple experiment to further characterize the impact of cell-specific IR signaling on glucose metabolism in the hippocampus.

7.5.2 Elucidating the Kinetics of Hippocampal GLUTs

Another avenue of interest involves the investigation of GLUT proteins specifically. As of today, there are 14 known GLUT subtypes, 10 of which have been detected in the CNS [324]. This number has risen significantly from the 4 or 5 originally characterized many years ago. As these GLUT sub-types are relatively homologous in terms of function and as many went undetected for a significant amount of time, it is not unreasonable to propose that we may have not yet discovered additional GLUTs that could mediate glucose metabolism under specific conditions. One potential way to explore this hypothesis involves an understanding of the kinetic properties of glucose transport. Of the three most predominant GLUTs in the brain, GLUT3 has the highest affinity, with K_m = ~3 mM compared to GLUT1 (K_m = ~20 mM) and GLUT4 (K_m = ~6 mM) [411-413]. Our glucose imaging protocol only uses 200 µM of 2-NBDG, which is significantly lower than the K_m for either GLUT3 or GLUT4. With such a low concentration of substrate in each dish during imaging, there is a small possibility that neither GLUT3 nor GLUT4 are activated under these conditions due to their much larger K_m values. However, as we detected significant increases in both 2-NBDG uptake and rates of utilization that paralleled the elevation in GLUT3 expression measured on our Western immunoblot assays, it is likely that the activated GLUT mediating these effects were indeed the GLUT3 subtype; yet, if there do exist yet uncharacterized GLUTs that are capable of binding in situations of low circulating glucose, such as during hypometabolism or hypoglycemia, or if these GLUTs were able to respond to much lower levels of IR signaling, as seen in the aged or AD brain, it could be that novel transporters are influencing glucose metabolism following sustained IR activity or INI administration.

Clearly, we still have a substantial way to go before fully elucidate the connection between brain IR activation and glucose metabolism. Harnessing these mechanisms as a therapeutic target would provide a significant advantage to our attempts at circumventing age- and/or AD-related cognitive decline. It is my hope that in the future, my work presented here will aid in these discoveries and advance our field in ways that impact not only the basic science community, but our partners in the clinic as well.

7.6 Conclusions

In conclusion, the work I completed during my dissertation substantially supports prior evidence of insulin's beneficial impact on learning and memory processes and provides insight into novel mechanisms mediating these effects in hippocampal neurons. While the study of insulin actions in the brain may still be relatively new, I feel that the worked presented here contributes well to our current body of knowledge and will in turn help future investigators in their attempt at elucidating these complex processes. As the field moves forward, so too will the clinical application of these discoveries, leading to improved therapies targeting age- and AD-related cognitive decline and granting a better quality of life to those afflicted by these pathologies.

APPENDICES

APPENDIX 1. LIST OF ABBREVIATIONS

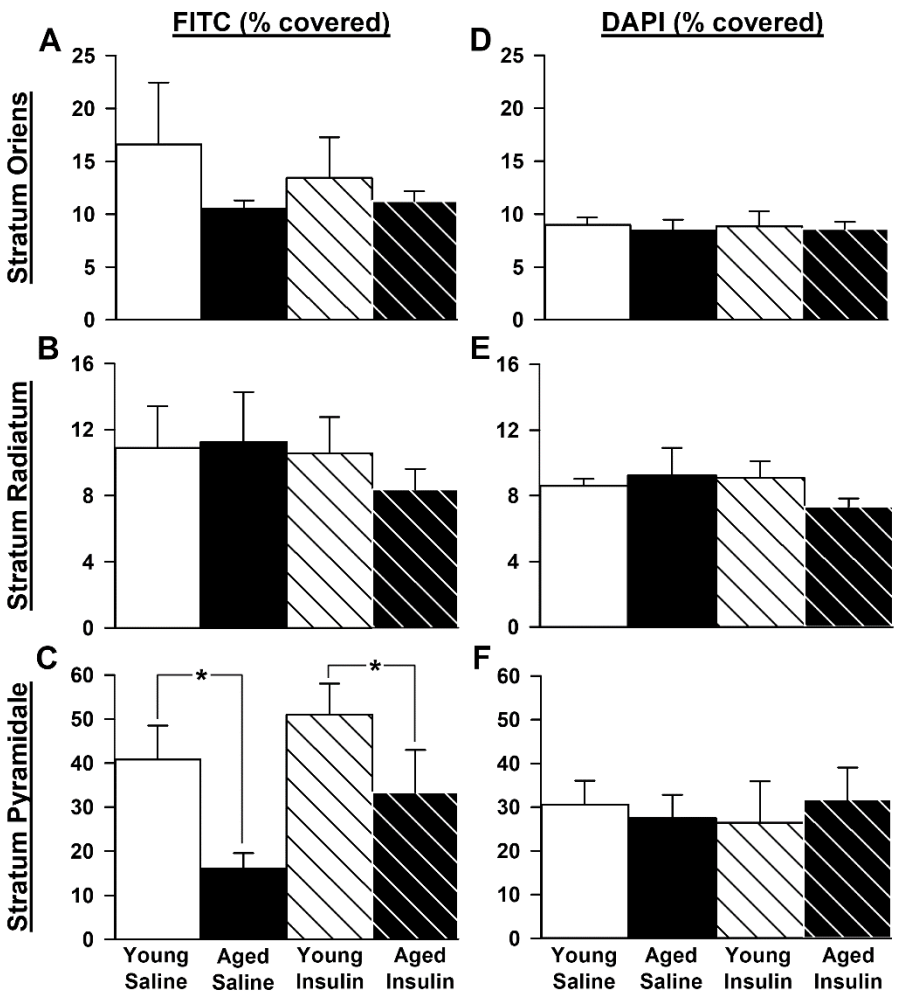
2-NBDG	2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose
^{3}H	tritium
3xTG-AD	triple transgenic-AD (AD mouse model)
AAV	adeno-associated virus
AD	Alzheimer's disease
ADAS-cog	Alzheimer's Disease Assessment Scale-cognitive subscale
ADCS-ADL	Alzheimer's Disease Cooperative Study – Activities of Daily Living
AHP/sAHP	afterhyperpolarization/slow afterhyperpolarization
AKT	protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
APOE	apolipoprotein E
APP/PS1	amyloid-precursor protein/human presenilin 1 (AD mouse model)
Aβ	amyloid beta
BBB	blood-brain barrier
BCA	bicinchoninic acid assay
C57Bl/6	C57 black 6 (WT/control mouse model)
CA1/2/3	*Cornu Ammonis* area 1/area 2/area 3
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CBF	cerebral blood flow

CICR	calcium-induced calcium release
C-peptide	connecting peptide
CSF	cerebrospinal fluid
DAPI	4′,6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DG	dentate gyrus
DIV	days *in vitro*
DNA	deoxyribonucleic acid
E18/19	embryonic day 18/19
EC	entorhinal cortex
EDTA	ethylenediaminetetraacetic acid
EGTA	egtazic acid
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
F344	Fisher 344
FDG-PET	fluorodeoxyglucose-positron emission tomography
FDUR	5-fluorodeoxyuridine/floxuridine
FITC	fluorescein isothiocyanate
FOV	field-of-view
$GABA_A$	ionotropic γ-aminobutyric acid (GABA) receptor
GLUT	glucose transporter
GRB2	growth factor receptor-bound protein 2

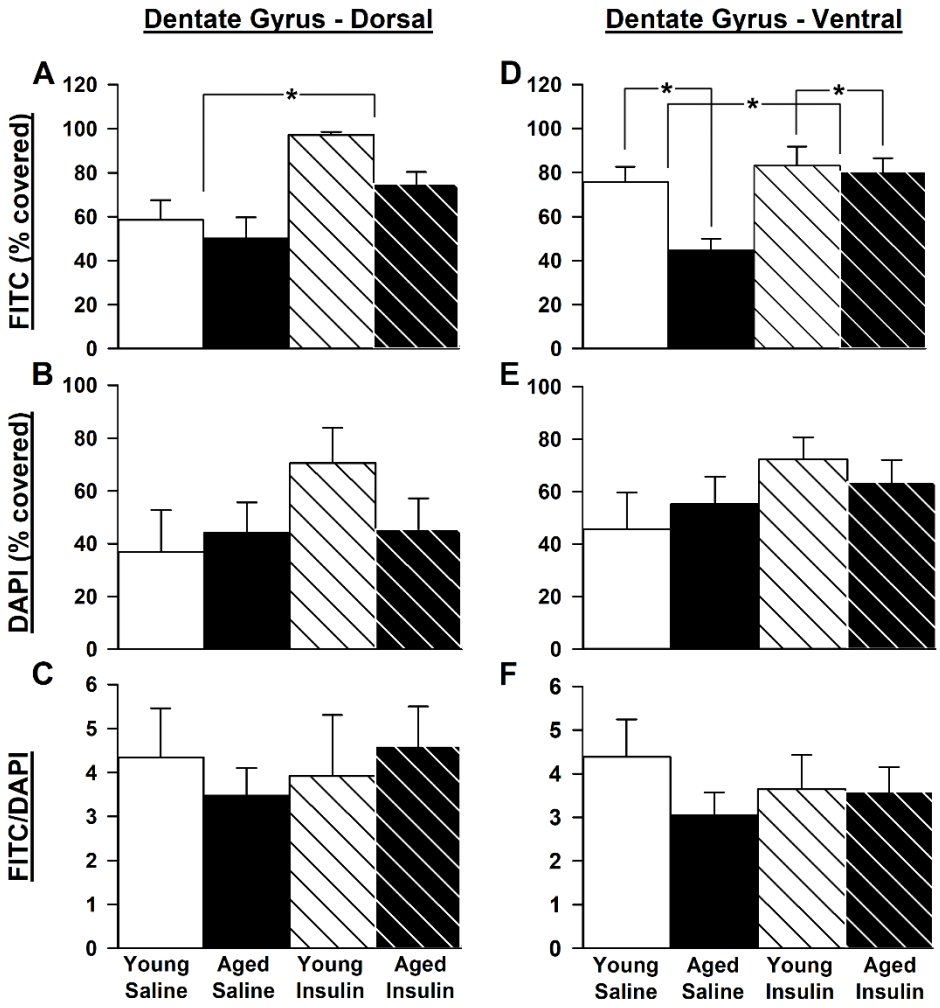
GSK3β	glycogen synthase kinase 3 beta
GSV	GLUT4 storage vesicle
HB	homogenizing buffer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high-fat diet
HFFD	high-fat-high-fructose diet
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HVA	high-voltage activated
ICC	immunocytochemistry
ICV	intracerebroventricular
IDE	insulin degrading enzyme
IF	immunofluorescence
IGF-I	insulin-like growth factor 1
IHC	immunohistochemistry
INI	intranasal insulin
IR	insulin receptor
IRS-1	insulin receptor substrate 1
IU	international units
IV	intravenous
LPS	lipopolysaccharide
LRP1	LDL receptor-related protein 1
LTP	long-term potentiation
LVA	low-voltage activated

MAPK	mitogen-activated protein kinase
MCI	mild-cognitive impairment
MEK	mitogen-activated protein kinase 1
MEM/SMEM	minimum/supplemented minimum essential medium
MRS	magnetic resonance spectroscopy
mTOR	mammalian target of rapamycin
MWM	Morris water maze
MΩ	megaohms
NMDA	N-methyl-D-aspartic acid
pA	picoamps
PDK1	pyruvate dehydrogenase kinase 1
pF	picofarad
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PKC	protein kinase C
PSD	post-synaptic density
PSD95	post-synaptic density protein 95
RIN	RNA integrity number
RIPA buffer	radioimmunoprecipitation assay buffer
r_m	membrane resistance
RNA	ribonucleic acid
ROI	region-of-interest

RyR	ryanodine receptor
SAMP8	Senescence Accelerated Mouse-Prone 8
SOS	son-of-sevenless
STZ	streptozotocin
T2DM	Type-2 diabetes mellitus
U	units
VGCC	voltage-gated calcium channel
Vm	membrane potential
WGA-HRP	wheat germ agglutinin-horseradish peroxidase
WT	wild-type
ZDF rat	Zucker diabetic fatty rat



Supplemental Figure 2.1 IR immunofluorescence quantification for FITC and DAPI channels across hippocampal subfields. IR immunofluorescence quantification for FITC and DAPI channels across hippocampal subfields. Data are presented as percent area covered in the ROI (% covered). (**A-C**) Immunopositive FITC fluorescence. (**D-F**) Immunopositive DAPI fluorescence. FITC/DAPI was reduced with age ($p < 0.05$). DAPI signals were not different across groups ($p > 0.05$). Data represent mean ± SEM. Asterisks (*) represent significance at $p < 0.05$.



Supplemental Figure 2.2 IR immunofluorescence quantification for FITC and DAPI in the dentate gyrus. IR immunofluorescence quantification for FITC and DAPI channels in the dentate gyrus. Data are presented as percent area covered in the ROI (% covered). (**A-C**) Dorsal blade of the DG was used to quantify the FITC channel (top), the DAPI channel (middle), and the ratio of the two (bottom). (**D-F**) Ventral blade of the DG was used to quantify the FITC channel (top), the DAPI channel (middle), and the ratio of the two (bottom). Data are presented as means ± SEM. Asterisks (*) represent significance at $p < 0.05$.

REFERENCES

1. Banting, F.G., et al., *Pancreatic Extracts in the Treatment of Diabetes Mellitus.* Can Med Assoc J, 1922. **12**(3): p. 141-6.
2. Chang, L., S.H. Chiang, and A.R. Saltiel, *Insulin signaling and the regulation of glucose transport.* Mol Med, 2004. **10**(7-12): p. 65-71.
3. Saltiel, A.R. and J.E. Pessin, *Insulin signaling in microdomains of the plasma membrane.* Traffic, 2003. **4**(11): p. 711-6.
4. Wilcox, G., *Insulin and insulin resistance.* Clin Biochem Rev, 2005. **26**(2): p. 19-39.
5. Bell, G.I., et al., *Sequence of the human insulin gene.* Nature, 1980. **284**(5751): p. 26-32.
6. Hou, J.C., L. Min, and J.E. Pessin, *Insulin granule biogenesis, trafficking and exocytosis.* Vitam Horm, 2009. **80**: p. 473-506.
7. Bratanova-Tochkova, T.K., et al., *Triggering and augmentation mechanisms, granule pools, and biphasic insulin secretion.* Diabetes, 2002. **51 Suppl 1**: p. S83-90.
8. Clot, J.P., et al., *Characterization of insulin degradation products generated in liver endosomes: in vivo and in vitro studies.* Mol Cell Endocrinol, 1990. **72**(3): p. 175-85.
9. Pell, M.E., W.C. Duckworth, and D.E. Peavy, *Localization of insulin degradation products to an intracellular site in isolated rat hepatocytes.* Biochem Biophys Res Commun, 1986. **137**(3): p. 1034-40.
10. Knutson, V.P., *Cellular trafficking and processing of the insulin receptor.* FASEB J, 1991. **5**(8): p. 2130-8.
11. Duckworth, W.C., R.G. Bennett, and F.G. Hamel, *Insulin degradation: progress and potential.* Endocr Rev, 1998. **19**(5): p. 608-24.
12. Belfiore, A., et al., *Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease.* Endocr Rev, 2009. **30**(6): p. 586-623.
13. Mosthaf, L., et al., *Functionally distinct insulin receptors generated by tissue-specific alternative splicing.* EMBO J, 1990. **9**(8): p. 2409-13.
14. Lee, J. and P.F. Pilch, *The insulin receptor: structure, function, and signaling.* Am J Physiol, 1994. **266**(2 Pt 1): p. C319-34.
15. Berhanu, P., et al., *Insulin receptors in isolated human adipocytes. Characterization by photoaffinity labeling and evidence for internalization and cellular processing.* J Clin Invest, 1983. **72**(6): p. 1958-70.
16. Pillay, T.S. and M.W. Makgoba, *Molecular mechanisms of insulin resistance.* S Afr Med J, 1991. **79**(10): p. 607-13.
17. Shepherd, P.R., *Mechanisms regulating phosphoinositide 3-kinase signalling in insulin-sensitive tissues.* Acta Physiol Scand, 2005. **183**(1): p. 3-12.
18. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.* J Biol Chem, 1998. **273**(22): p. 13375-8.

19. Solinas, G., et al., *Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates.* Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16454-9.
20. Inoue, M., et al., *The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin.* Nature, 2003. **422**(6932): p. 629-33.
21. Marshall, S. and J.M. Olefsky, *Effects of insulin incubation on insulin binding, glucose transport, and insulin degradation by isolated rat adipocytes. Evidence for hormone-induced desensitization at the receptor and postreceptor level.* J Clin Invest, 1980. **66**(4): p. 763-72.
22. Ciaraldi, T., et al., *Insulin receptors on cultured hypothalamic cells: functional and structural differences from receptors on peripheral target cells.* Endocrinology, 1985. **116**(6): p. 2179-85.
23. Ide, R., et al., *High glucose condition desensitizes insulin action at the levels of receptor kinase.* Endocr J, 1995. **42**(1): p. 1-8.
24. Yuan, L., R. Ziegler, and A. Hamann, *Chronic hyperinsulinism induced down-regulation of insulin post-receptor signaling transduction in Hep G2 cells.* J Huazhong Univ Sci Technolog Med Sci, 2002. **22**(4): p. 313-6.
25. Werner, E.D., et al., *Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302.* J Biol Chem, 2004. **279**(34): p. 35298-305.
26. Pirola, L., et al., *Phosphoinositide 3-kinase-mediated reduction of insulin receptor substrate-1/2 protein expression via different mechanisms contributes to the insulin-induced desensitization of its signaling pathways in L6 muscle cells.* J Biol Chem, 2003. **278**(18): p. 15641-51.
27. Bertacca, A., et al., *Continually high insulin levels impair Akt phosphorylation and glucose transport in human myoblasts.* Metabolism, 2005. **54**(12): p. 1687-93.
28. Tanti, J.F. and J. Jager, *Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation.* Curr Opin Pharmacol, 2009. **9**(6): p. 753-62.
29. Geijselaers, S.L.C., et al., *Glucose regulation, cognition, and brain MRI in type 2 diabetes: a systematic review.* Lancet Diabetes Endocrinol, 2015. **3**(1): p. 75-89.
30. Biessels, G.J., et al., *Ageing and diabetes: implications for brain function.* Eur J Pharmacol, 2002. **441**(1-2): p. 1-14.
31. Messier, C. and K. Teutenberg, *The role of insulin, insulin growth factor, and insulin-degrading enzyme in brain aging and Alzheimer's disease.* Neural Plast, 2005. **12**(4): p. 311-28.
32. Mayeda, E.R., R.A. Whitmer, and K. Yaffe, *Diabetes and cognition.* Clin Geriatr Med, 2015. **31**(1): p. 101-15, ix.
33. Cholerton, B., L.D. Baker, and S. Craft, *Insulin, cognition, and dementia.* Eur J Pharmacol, 2013. **719**(1-3): p. 170-9.
34. Zhao, W.Q., et al., *Insulin and the insulin receptor in experimental models of learning and memory.* Eur J Pharmacol, 2004. **490**(1-3): p. 71-81.
35. Tilvis, R.S., et al., *Predictors of cognitive decline and mortality of aged people over a 10-year period.* J Gerontol A Biol Sci Med Sci, 2004. **59**(3): p. 268-74.
36. Kuusisto, J., et al., *Essential hypertension and cognitive function. The role of hyperinsulinemia.* Hypertension, 1993. **22**(5): p. 771-9.

37. Burns, J.M., et al., *Insulin is differentially related to cognitive decline and atrophy in Alzheimer's disease and aging.* Biochim Biophys Acta, 2012. **1822**(3): p. 333-9.
38. Baker, L.D., et al., *Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes.* Arch Neurol, 2011. **68**(1): p. 51-7.
39. Soininen, H., et al., *Diabetes mellitus and brain atrophy: a computed tomography study in an elderly population.* Neurobiol Aging, 1992. **13**(6): p. 717-21.
40. Convit, A., et al., *Reduced glucose tolerance is associated with poor memory performance and hippocampal atrophy among normal elderly.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 2019-22.
41. Araki, Y., et al., *MRI of the brain in diabetes mellitus.* Neuroradiology, 1994. **36**(2): p. 101-3.
42. Heijer, T., et al., *Association between blood pressure levels over time and brain atrophy in the elderly.* Neurobiol Aging, 2003. **24**(2): p. 307-13.
43. Ott, A., et al., *Association of diabetes mellitus and dementia: the Rotterdam Study.* Diabetologia, 1996. **39**(11): p. 1392-7.
44. Stolk, R.P., et al., *Insulin and cognitive function in an elderly population. The Rotterdam Study.* Diabetes Care, 1997. **20**(5): p. 792-5.
45. Craft, S., et al., *Effects of hyperglycemia on memory and hormone levels in dementia of the Alzheimer type: a longitudinal study.* Behav Neurosci, 1993. **107**(6): p. 926-40.
46. Bucht, G., et al., *Changes in blood glucose and insulin secretion in patients with senile dementia of Alzheimer type.* Acta Med Scand, 1983. **213**(5): p. 387-92.
47. Peila, R., et al., *Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: The Honolulu-Asia Aging Study.* Diabetes, 2002. **51**(4): p. 1256-62.
48. de la Monte, S.M. and J.R. Wands, *Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease.* J Alzheimers Dis, 2005. **7**(1): p. 45-61.
49. Arnold, S.E., et al., *Brain insulin resistance in type 2 diabetes and Alzheimer disease: concepts and conundrums.* Nat Rev Neurol, 2018. **14**(3): p. 168-181.
50. Arnold, S.E., et al., *High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice.* Neurobiol Dis, 2014. **67**: p. 79-87.
51. Baskin, D.G., et al., *Genetically obese Zucker rats have abnormally low brain insulin content.* Life Sci, 1985. **36**(7): p. 627-33.
52. Liu, Z., et al., *High-fat diet induces hepatic insulin resistance and impairment of synaptic plasticity.* PLoS One, 2015. **10**(5): p. e0128274.
53. Calvo-Ochoa, E., et al., *Short-term high-fat-and-fructose feeding produces insulin signaling alterations accompanied by neurite and synaptic reduction and astroglial activation in the rat hippocampus.* J Cereb Blood Flow Metab, 2014. **34**(6): p. 1001-8.
54. Battu, C.E., et al., *Alterations of PI3K and Akt signaling pathways in the hippocampus and hypothalamus of Wistar rats treated with highly palatable food.* Nutr Neurosci, 2012. **15**(1): p. 10-7.

55. Pancani, T., et al., *Effect of high-fat diet on metabolic indices, cognition, and neuronal physiology in aging F344 rats.* Neurobiol Aging, 2013. **34**(8): p. 1977-87.
56. Kothari, V., et al., *High fat diet induces brain insulin resistance and cognitive impairment in mice.* Biochim Biophys Acta, 2017. **1863**(2): p. 499-508.
57. Bhat, N.R. and L. Thirumangalakudi, *Increased tau phosphorylation and impaired brain insulin/IGF signaling in mice fed a high fat/high cholesterol diet.* J Alzheimers Dis, 2013. **36**(4): p. 781-9.
58. Salkovic-Petrisic, M., et al., *What have we learned from the streptozotocin-induced animal model of sporadic Alzheimer's disease, about the therapeutic strategies in Alzheimer's research.* J Neural Transm (Vienna), 2013. **120**(1): p. 233-52.
59. Biessels, G.J., et al., *Place learning and hippocampal synaptic plasticity in streptozotocin-induced diabetic rats.* Diabetes, 1996. **45**(9): p. 1259-66.
60. Biessels, G.J., et al., *Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: effects of insulin treatment.* Brain Res, 1998. **800**(1): p. 125-35.
61. Kamal, A., et al., *Learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: interaction of diabetes and ageing.* Diabetologia, 2000. **43**(4): p. 500-6.
62. Kamal, A., et al., *Hippocampal synaptic plasticity in streptozotocin-diabetic rats: impairment of long-term potentiation and facilitation of long-term depression.* Neuroscience, 1999. **90**(3): p. 737-45.
63. Biessels, G.J., et al., *Neurophysiological changes in the central and peripheral nervous system of streptozotocin-diabetic rats. Course of development and effects of insulin treatment.* Brain, 1999. **122 (Pt 4)**: p. 757-68.
64. Abdul-Rahman, O., et al., *Altered gene expression profiles in the hippocampus and prefrontal cortex of type 2 diabetic rats.* BMC Genomics, 2012. **13**: p. 81.
65. Gispen, W.H. and G.J. Biessels, *Cognition and synaptic plasticity in diabetes mellitus.* Trends Neurosci, 2000. **23**(11): p. 542-9.
66. Woods, S.C. and D. Porte, Jr., *Effect of intracisternal insulin on plasma glucose and insulin in the dog.* Diabetes, 1975. **24**(10): p. 905-9.
67. Chowers, I., S. Lavy, and L. Halpern, *Effect of insulin administered intracisternally on the glucose level of the blood and the cerebrospinal fluid in vagotomized dogs.* Exp Neurol, 1966. **14**(3): p. 383-9.
68. Phillips, M.E. and R.V. Coxon, *Effect of insulin and phenobarbital on uptake of 2-deoxyglucose by brain slices and hemidiaphragms.* J Neurochem, 1976. **27**(2): p. 643-5.
69. Havrankova, J., J. Roth, and M. Brownstein, *Insulin receptors are widely distributed in the central nervous system of the rat.* Nature, 1978. **272**(5656): p. 827-9.
70. Havrankova, J., J. Roth, and M.J. Brownstein, *Concentrations of insulin and insulin receptors in the brain are independent of peripheral insulin levels. Studies of obese and streptozotocin-treated rodents.* J Clin Invest, 1979. **64**(2): p. 636-42.
71. Havrankova, J., et al., *Identification of insulin in rat brain.* Proc Natl Acad Sci U S A, 1978. **75**(11): p. 5737-41.

72. Havrankova, J., M. Brownstein, and J. Roth, *Insulin and insulin receptors in rodent brain.* Diabetologia, 1981. **20**(Suppl 1): p. 268-273.
73. Banks, W.A., A.J. Kastin, and W. Pan, *Uptake and degradation of blood-borne insulin by the olfactory bulb.* Peptides, 1999. **20**(3): p. 373-8.
74. Baskin, D.G., et al., *Insulin in the brain.* Annu Rev Physiol, 1987. **49**: p. 335-47.
75. Dore, S., et al., *Distribution and levels of [125I]IGF-I, [125I]IGF-II and [125I]insulin receptor binding sites in the hippocampus of aged memory-unimpaired and -impaired rats.* Neuroscience, 1997. **80**(4): p. 1033-40.
76. Kar, S., J.G. Chabot, and R. Quirion, *Quantitative autoradiographic localization of [125I]insulin-like growth factor I, [125I]insulin-like growth factor II, and [125I]insulin receptor binding sites in developing and adult rat brain.* J Comp Neurol, 1993. **333**(3): p. 375-97.
77. Marks, J.L., M.G. King, and D.G. Baskin, *Localization of insulin and type 1 IGF receptors in rat brain by in vitro autoradiography and in situ hybridization.* Adv Exp Med Biol, 1991. **293**: p. 459-70.
78. Unger, J.W., J.N. Livingston, and A.M. Moss, *Insulin receptors in the central nervous system: localization, signalling mechanisms and functional aspects.* Prog Neurobiol, 1991. **36**(5): p. 343-62.
79. Unger, J., et al., *Distribution of insulin receptor-like immunoreactivity in the rat forebrain.* Neuroscience, 1989. **31**(1): p. 143-57.
80. Clarke, D.W., et al., *Insulin binds to specific receptors and stimulates 2-deoxy-D-glucose uptake in cultured glial cells from rat brain.* J Biol Chem, 1984. **259**(19): p. 11672-5.
81. Garwood, C.J., et al., *Insulin and IGF1 signalling pathways in human astrocytes in vitro and in vivo; characterisation, subcellular localisation and modulation of the receptors.* Mol Brain, 2015. **8**: p. 51.
82. Schwartz, M.W., et al., *Insulin in the brain: a hormonal regulator of energy balance.* Endocr Rev, 1992. **13**(3): p. 387-414.
83. Heni, M., et al., *Insulin promotes glycogen storage and cell proliferation in primary human astrocytes.* PLoS One, 2011. **6**(6): p. e21594.
84. Raizada, M.K., J.W. Yang, and R.E. Fellows, *Binding of [125I]insulin to specific receptors and stimulation of nucleotide incorporation in cells cultured from rat brain.* Brain Res, 1980. **200**(2): p. 389-400.
85. Gammeltoft, S., et al., *Insulin receptors in rat brain: insulin stimulates phosphorylation of its receptor beta-subunit.* FEBS Lett, 1984. **172**(1): p. 87-90.
86. Boyd, F.T., Jr. and M.K. Raizada, *Effects of insulin and tunicamycin on neuronal insulin receptors in culture.* Am J Physiol, 1983. **245**(3): p. C283-7.
87. Apostolatos, A., et al., *Insulin promotes neuronal survival via the alternatively spliced protein kinase CdeltaII isoform.* J Biol Chem, 2012. **287**(12): p. 9299-310.
88. Banks, W.A., J.B. Owen, and M.A. Erickson, *Insulin in the brain: there and back again.* Pharmacol Ther, 2012. **136**(1): p. 82-93.
89. Blazquez, E., et al., *Insulin in the brain: its pathophysiological implications for States related with central insulin resistance, type 2 diabetes and Alzheimer's disease.* Front Endocrinol (Lausanne), 2014. **5**: p. 161.

90. Ferrario, C.R. and L.P. Reagan, *Insulin-mediated synaptic plasticity in the CNS: Anatomical, functional and temporal contexts.* Neuropharmacology, 2018. **136**(Pt B): p. 182-191.
91. Lee, S.H., et al., *Insulin in the nervous system and the mind: Functions in metabolism, memory, and mood.* Mol Metab, 2016. **5**(8): p. 589-601.
92. Huang, C.C., et al., *Insulin induces a novel form of postsynaptic mossy fiber long-term depression in the hippocampus.* Mol Cell Neurosci, 2003. **24**(3): p. 831-41.
93. Zhao, W., et al., *Permissive role of insulin in the expression of long-term potentiation in the hippocampus of immature rats.* Neurosignals, 2010. **18**(4): p. 236-45.
94. Frazier, H.N., et al., *Expression of a Constitutively Active Human Insulin Receptor in Hippocampal Neurons Does Not Alter VGCC Currents.* Neurochem Res, 2018. **44**(1): p. 269-280.
95. Frazier, H.N., et al., *Long-term intranasal insulin aspart: a profile of gene expression, memory, and insulin receptors in aged F344 rats.* J Gerontol A Biol Sci Med Sci, 2019.
96. Nemoto, T., et al., *New insights concerning insulin synthesis and its secretion in rat hippocampus and cerebral cortex: amyloid-beta1-42-induced reduction of proinsulin level via glycogen synthase kinase-3beta.* Cell Signal, 2014. **26**(2): p. 253-9.
97. Ghasemi, R., et al., *Insulin in the brain: sources, localization and functions.* Mol Neurobiol, 2013. **47**(1): p. 145-71.
98. Margolis, R.U. and N. Altszuler, *Insulin in the cerebrospinal fluid.* Nature, 1967. **215**(5108): p. 1375-6.
99. Banks, W.A., et al., *Transport of insulin across the blood-brain barrier: saturability at euglycemic doses of insulin.* Peptides, 1997b. **18**(9): p. 1423-9.
100. Banks, W.A., *The source of cerebral insulin.* Eur J Pharmacol, 2004. **490**(1-3): p. 5-12.
101. Baskin, D.G., et al., *Quantitative autoradiographic evidence for insulin receptors in the choroid plexus of the rat brain.* Diabetes, 1986. **35**(2): p. 246-9.
102. Baskin, D.G., et al., *Regional concentrations of insulin in the rat brain.* Endocrinology, 1983. **112**(3): p. 898-903.
103. Werther, G.A., et al., *Localization and characterization of insulin receptors in rat brain and pituitary gland using in vitro autoradiography and computerized densitometry.* Endocrinology, 1987. **121**(4): p. 1562-70.
104. Kaiyala, K.J., et al., *Obesity induced by a high-fat diet is associated with reduced brain insulin transport in dogs.* Diabetes, 2000. **49**(9): p. 1525-33.
105. Baura, G.D., et al., *Insulin transport from plasma into the central nervous system is inhibited by dexamethasone in dogs.* Diabetes, 1996. **45**(1): p. 86-90.
106. Israel, P.A., et al., *Effect of diet-induced obesity and experimental hyperinsulinemia on insulin uptake into CSF of the rat.* Brain Res Bull, 1993. **30**(5-6): p. 571-5.
107. Stein, L.J., et al., *Reduced effect of experimental peripheral hyperinsulinemia to elevate cerebrospinal fluid insulin concentrations of obese Zucker rats.* Endocrinology, 1987. **121**(5): p. 1611-5.
108. Banks, W.A., *The dam breaks: disruption of the blood-brain barrier in diabetes mellitus.* Am J Physiol Heart Circ Physiol, 2006. **291**(6): p. H2595-6.

109. Banks, W.A., J.B. Jaspan, and A.J. Kastin, *Effect of diabetes mellitus on the permeability of the blood-brain barrier to insulin.* Peptides, 1997c. **18**(10): p. 1577-84.
110. Huber, J.D., R.L. VanGilder, and K.A. Houser, *Streptozotocin-induced diabetes progressively increases blood-brain barrier permeability in specific brain regions in rats.* Am J Physiol Heart Circ Physiol, 2006. **291**(6): p. H2660-8.
111. Chehade, J.M., M.J. Haas, and A.D. Mooradian, *Diabetes-related changes in rat cerebral occludin and zonula occludens-1 (ZO-1) expression.* Neurochem Res, 2002. **27**(3): p. 249-52.
112. Abbott, M.A., D.G. Wells, and J.R. Fallon, *The insulin receptor tyrosine kinase substrate p58/53 and the insulin receptor are components of CNS synapses.* J Neurosci, 1999. **19**(17): p. 7300-8.
113. Adzovic, L. and L. Domenici, *Insulin induces phosphorylation of the AMPA receptor subunit GluR1, reversed by ZIP, and over-expression of Protein Kinase M zeta, reversed by amyloid beta.* J Neurochem, 2014. **131**(5): p. 582-7.
114. Zhao, W., et al., *Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats.* J Biol Chem, 1999. **274**(49): p. 34893-902.
115. Pearson-Leary, J., et al., *Insulin modulates hippocampally-mediated spatial working memory via glucose transporter-4.* Behav Brain Res, 2018. **338**: p. 32-39.
116. Chen, Y., et al., *Intranasal Insulin Ameliorates Cerebral Hypometabolism, Neuronal Loss, and Astrogliosis in Streptozotocin-Induced Alzheimer's Rat Model.* Neurotox Res, 2018. **33**(4): p. 716-724.
117. Vogt, M.C. and J.C. Bruning, *CNS insulin signaling in the control of energy homeostasis and glucose metabolism - from embryo to old age.* Trends Endocrinol Metab, 2013. **24**(2): p. 76-84.
118. Hirvonen, J., et al., *Effects of insulin on brain glucose metabolism in impaired glucose tolerance.* Diabetes, 2011. **60**(2): p. 443-7.
119. Agrawal, R., et al., *Insulin receptor signaling in rat hippocampus: a study in STZ (ICV) induced memory deficit model.* Eur Neuropsychopharmacol, 2011. **21**(3): p. 261-73.
120. Tchilian, E.Z., et al., *125I-insulin binding is decreased in olfactory bulbs of aged rats.* Neuropeptides, 1990. **17**(4): p. 193-6.
121. Zaia, A. and L. Piantanelli, *Alterations of brain insulin receptor characteristics in aging mice.* Arch Gerontol Geriatr, 1996. **23**(1): p. 27-37.
122. Frazier, H.N., et al., *Broadening the definition of brain insulin resistance in aging and Alzheimer's disease.* Exp Neurol, 2019. **313**: p. 79-87.
123. Akintola, A.A. and D. van Heemst, *Insulin, aging, and the brain: mechanisms and implications.* Front Endocrinol (Lausanne), 2015. **6**: p. 13.
124. Belanger, A., et al., *Preserved LTP and water maze learning in hyperglycaemic-hyperinsulinemic ZDF rats.* Physiol Behav, 2004. **83**(3): p. 483-94.
125. Stanley, M., et al., *The Effects of Peripheral and Central High Insulin on Brain Insulin Signaling and Amyloid-beta in Young and Old APP/PS1 Mice.* J Neurosci, 2016. **36**(46): p. 11704-11715.

126. Maimaiti, S., et al., *Intranasal Insulin Improves Age-Related Cognitive Deficits and Reverses Electrophysiological Correlates of Brain Aging.* J Gerontol A Biol Sci Med Sci, 2016. **71**(1): p. 30-9.
127. Anderson, K.L., et al., *Impact of Single or Repeated Dose Intranasal Zinc-free Insulin in Young and Aged F344 Rats on Cognition, Signaling, and Brain Metabolism.* J Gerontol A Biol Sci Med Sci, 2017. **72**(2): p. 189-197.
128. Sartorius, T., et al., *The brain response to peripheral insulin declines with age: a contribution of the blood-brain barrier?* PLoS One, 2015. **10**(5): p. e0126804.
129. Frolich, L., et al., *Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease.* J Neural Transm (Vienna), 1998. **105**(4-5): p. 423-38.
130. Craft, S., et al., *Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype.* Neurology, 1998. **50**(1): p. 164-8.
131. Hoyer, S., *Is sporadic Alzheimer disease the brain type of non-insulin dependent diabetes mellitus? A challenging hypothesis.* J Neural Transm (Vienna), 1998. **105**(4-5): p. 415-22.
132. Steen, E., et al., *Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes?* J Alzheimers Dis, 2005. **7**(1): p. 63-80.
133. Bedse, G., et al., *Aberrant insulin signaling in Alzheimer's disease: current knowledge.* Front Neurosci, 2015. **9**: p. 204.
134. Craft, S., *Insulin resistance syndrome and Alzheimer's disease: age- and obesity-related effects on memory, amyloid, and inflammation.* Neurobiol Aging, 2005. **26 Suppl 1**: p. 65-9.
135. Hoyer, S., *The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: an update.* J Neural Transm (Vienna), 2002. **109**(3): p. 341-60.
136. Molina, J.A., et al., *Cerebrospinal fluid levels of insulin in patients with Alzheimer's disease.* Acta Neurol Scand, 2002. **106**(6): p. 347-50.
137. Schubert, M., et al., *Role for neuronal insulin resistance in neurodegenerative diseases.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3100-5.
138. Arab, L., et al., *Consequences of Aberrant Insulin Regulation in the Brain: Can Treating Diabetes be Effective for Alzheimer's Disease.* Curr Neuropharmacol, 2011. **9**(4): p. 693-705.
139. Chen, Z. and C. Zhong, *Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies.* Prog Neurobiol, 2013. **108**: p. 21-43.
140. Lee, J.H., et al., *Targeting Insulin for Alzheimer's Disease: Mechanisms, Status and Potential Directions.* J Alzheimers Dis, 2018.
141. Benedict, C. and C.A. Grillo, *Insulin Resistance as a Therapeutic Target in the Treatment of Alzheimer's Disease: A State-of-the-Art Review.* Front Neurosci, 2018. **12**: p. 215.
142. Wallum, B.J., et al., *Cerebrospinal fluid insulin levels increase during intravenous insulin infusions in man.* J Clin Endocrinol Metab, 1987. **64**(1): p. 190-4.
143. Craft, S., et al., *Enhancement of memory in Alzheimer disease with insulin and somatostatin, but not glucose.* Arch Gen Psychiatry, 1999. **56**(12): p. 1135-40.

144. Craft, S., et al., *Memory improvement following induced hyperinsulinemia in Alzheimer's disease.* Neurobiol Aging, 1996. **17**(1): p. 123-30.
145. Craft, S., et al., *Insulin dose-response effects on memory and plasma amyloid precursor protein in Alzheimer's disease: interactions with apolipoprotein E genotype.* Psychoneuroendocrinology, 2003. **28**(6): p. 809-22.
146. Kern, W., et al., *Improving influence of insulin on cognitive functions in humans.* Neuroendocrinology, 2001. **74**(4): p. 270-80.
147. Watson, G.S., et al., *Insulin increases CSF Abeta42 levels in normal older adults.* Neurology, 2003. **60**(12): p. 1899-903.
148. Viswaprakash, N., et al., *Insulin treatment restores glutamate (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor function in the hippocampus of diabetic rats.* J Neurosci Res, 2015. **93**(9): p. 1442-50.
149. Schmidt, R.E., et al., *Analysis of the Zucker Diabetic Fatty (ZDF) type 2 diabetic rat model suggests a neurotrophic role for insulin/IGF-I in diabetic autonomic neuropathy.* Am J Pathol, 2003. **163**(1): p. 21-8.
150. Luchsinger, J.A., et al., *Hyperinsulinemia and risk of Alzheimer disease.* Neurology, 2004. **63**(7): p. 1187-92.
151. Manin, M., et al., *Chronic intracerebroventricular infusion of insulin failed to alter brain insulin-binding sites, food intake, and body weight.* J Neurochem, 1988. **51**(6): p. 1689-95.
152. Grillo, C.A., et al., *Insulin-stimulated translocation of GLUT4 to the plasma membrane in rat hippocampus is PI3-kinase dependent.* Brain Res, 2009. **1296**: p. 35-45.
153. Park, C.R., et al., *Intracerebroventricular insulin enhances memory in a passive-avoidance task.* Physiol Behav, 2000. **68**(4): p. 509-14.
154. Adzovic, L., et al., *Insulin improves memory and reduces chronic neuroinflammation in the hippocampus of young but not aged brains.* J Neuroinflammation, 2015. **12**: p. 63.
155. Canteiro, P.B., et al., *Insulin treatment protects the brain against neuroinflammation by reducing cerebral cytokines and modulating mitochondrial function.* Brain Res Bull, 2019. **149**: p. 120-128.
156. Haj-ali, V., G. Mohaddes, and S.H. Babri, *Intracerebroventricular insulin improves spatial learning and memory in male Wistar rats.* Behav Neurosci, 2009. **123**(6): p. 1309-14.
157. Thorne, R.G., et al., *Quantitative analysis of the olfactory pathway for drug delivery to the brain.* Brain Res, 1995. **692**(1-2): p. 278-82.
158. Liu, X.F., et al., *Intranasal administration of insulin-like growth factor-I bypasses the blood-brain barrier and protects against focal cerebral ischemic damage.* J Neurol Sci, 2001. **187**(1-2): p. 91-7.
159. Illum, L., *Transport of drugs from the nasal cavity to the central nervous system.* Eur J Pharm Sci, 2000. **11**(1): p. 1-18.
160. Thorne, R.G., et al., *Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration.* Neuroscience, 2004. **127**(2): p. 481-96.
161. Born, J., et al., *Sniffing neuropeptides: a transnasal approach to the human brain.* Nat Neurosci, 2002. **5**(6): p. 514-6.

162. Lochhead, J.J., et al., *Distribution of insulin in trigeminal nerve and brain after intranasal administration.* Sci Rep, 2019. **9**(1): p. 2621.
163. Kern, W., et al., *Central nervous system effects of intranasally administered insulin during euglycemia in men.* Diabetes, 1999. **48**(3): p. 557-63.
164. Reger, M.A., et al., *Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype.* Neurobiol Aging, 2006. **27**(3): p. 451-8.
165. Stockhorst, U., et al., *Insulin and the CNS: effects on food intake, memory, and endocrine parameters and the role of intranasal insulin administration in humans.* Physiol Behav, 2004. **83**(1): p. 47-54.
166. Dash, S., et al., *Intranasal insulin suppresses endogenous glucose production in humans compared with placebo in the presence of similar venous insulin concentrations.* Diabetes, 2015. **64**(3): p. 766-74.
167. Hallschmid, M., et al., *Intranasal insulin reduces body fat in men but not in women.* Diabetes, 2004. **53**(11): p. 3024-9.
168. Kullmann, S., et al., *Intranasal insulin modulates intrinsic reward and prefrontal circuitry of the human brain in lean women.* Neuroendocrinology, 2013. **97**(2): p. 176-82.
169. Thienel, M., et al., *Intranasal insulin decreases circulating cortisol concentrations during early sleep in elderly humans.* Neurobiol Aging, 2017. **54**: p. 170-174.
170. Benedict, C., et al., *Intranasal insulin improves memory in humans.* Psychoneuroendocrinology, 2004. **29**(10): p. 1326-34.
171. Craft, S., et al., *Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial.* Arch Neurol, 2012. **69**(1): p. 29-38.
172. Claxton, A., et al., *Long-acting intranasal insulin detemir improves cognition for adults with mild cognitive impairment or early-stage Alzheimer's disease dementia.* J Alzheimers Dis, 2015. **44**(3): p. 897-906.
173. Rosenbloom, M.H., et al., *A single-dose pilot trial of intranasal rapid-acting insulin in apolipoprotein E4 carriers with mild-moderate Alzheimer's disease.* CNS Drugs, 2014. **28**(12): p. 1185-9.
174. Benedict, C., et al., *Differential sensitivity of men and women to anorexigenic and memory-improving effects of intranasal insulin.* J Clin Endocrinol Metab, 2008. **93**(4): p. 1339-44.
175. Claxton, A., et al., *Sex and ApoE genotype differences in treatment response to two doses of intranasal insulin in adults with mild cognitive impairment or Alzheimer's disease.* J Alzheimers Dis, 2013. **35**(4): p. 789-97.
176. Schilling, T.M., et al., *Intranasal insulin increases regional cerebral blood flow in the insular cortex in men independently of cortisol manipulation.* Hum Brain Mapp, 2014. **35**(5): p. 1944-56.
177. Brunner, Y.F., et al., *Central insulin administration improves odor-cued reactivation of spatial memory in young men.* J Clin Endocrinol Metab, 2015. **100**(1): p. 212-9.
178. Novak, V., et al., *Enhancement of vasoreactivity and cognition by intranasal insulin in type 2 diabetes.* Diabetes Care, 2014. **37**(3): p. 751-9.

179. Akintola, A.A., et al., *Effect of intranasally administered insulin on cerebral blood flow and perfusion; a randomized experiment in young and older adults.* Aging (Albany NY), 2017. **9**(3): p. 790-802.
180. Benedict, C., et al., *Intranasal insulin as a therapeutic option in the treatment of cognitive impairments.* Exp Gerontol, 2011. **46**(2-3): p. 112-5.
181. Chapman, C.D., et al., *Intranasal insulin in Alzheimer's disease: Food for thought.* Neuropharmacology, 2018. **136**(Pt B): p. 196-201.
182. de la Monte, S.M., *Early intranasal insulin therapy halts progression of neurodegeneration: progress in Alzheimer's disease therapeutics.* Aging health, 2012. **8**(1): p. 61-64.
183. de la Monte, S.M., *Intranasal insulin therapy for cognitive impairment and neurodegeneration: current state of the art.* Expert Opin Drug Deliv, 2013. **10**(12): p. 1699-709.
184. Freiherr, J., et al., *Intranasal insulin as a treatment for Alzheimer's disease: a review of basic research and clinical evidence.* CNS Drugs, 2013. **27**(7): p. 505-14.
185. Lochhead, J.J. and R.G. Thorne, *Intranasal delivery of biologics to the central nervous system.* Adv Drug Deliv Rev, 2012. **64**(7): p. 614-28.
186. Strachan, M.W., *Insulin and cognitive function in humans: experimental data and therapeutic considerations.* Biochem Soc Trans, 2005. **33**(Pt 5): p. 1037-40.
187. Chen, Y., et al., *Intranasal insulin restores insulin signaling, increases synaptic proteins, and reduces Abeta level and microglia activation in the brains of 3xTg-AD mice.* Exp Neurol, 2014. **261**: p. 610-9.
188. Barone, E., et al., *Biliverdin Reductase-A Mediates the Beneficial Effects of Intranasal Insulin in Alzheimer Disease.* Mol Neurobiol, 2018.
189. Salameh, T.S., et al., *Central Nervous System Delivery of Intranasal Insulin: Mechanisms of Uptake and Effects on Cognition.* J Alzheimers Dis, 2015. **47**(3): p. 715-28.
190. Kamei, N., et al., *Effect of an Enhanced Nose-to-Brain Delivery of Insulin on Mild and Progressive Memory Loss in the Senescence-Accelerated Mouse.* Mol Pharm, 2017. **14**(3): p. 916-927.
191. Mao, Y.F., et al., *Intranasal insulin alleviates cognitive deficits and amyloid pathology in young adult APPswe/PS1dE9 mice.* Aging Cell, 2016. **15**(5): p. 893-902.
192. Bell, G.A. and D.A. Fadool, *Awake, long-term intranasal insulin treatment does not affect object memory, odor discrimination, or reversal learning in mice.* Physiol Behav, 2017. **174**: p. 104-113.
193. Zhang, Y., et al., *Intranasal Insulin Prevents Anesthesia-Induced Spatial Learning and Memory Deficit in Mice.* Sci Rep, 2016. **6**: p. 21186.
194. Brabazon, F., et al., *Intranasal insulin treatment of an experimental model of moderate traumatic brain injury.* J Cereb Blood Flow Metab, 2017. **37**(9): p. 3203-3218.
195. Beirami, E., et al., *Intranasal insulin treatment alleviates methamphetamine induced anxiety-like behavior and neuroinflammation.* Neurosci Lett, 2017. **660**: p. 122-129.

196. Mamik, M.K., et al., *Insulin Treatment Prevents Neuroinflammation and Neuronal Injury with Restored Neurobehavioral Function in Models of HIV/AIDS Neurodegeneration.* J Neurosci, 2016. **36**(41): p. 10683-10695.
197. Chistyakova, O.V., et al., *Intranasal administration of insulin eliminates the deficit of long-term spatial memory in rats with neonatal diabetes mellitus.* Dokl Biochem Biophys, 2011. **440**: p. 216-8.
198. Rajasekar, N., et al., *Intranasal Insulin Administration Ameliorates Streptozotocin (ICV)-Induced Insulin Receptor Dysfunction, Neuroinflammation, Amyloidogenesis, and Memory Impairment in Rats.* Mol Neurobiol, 2017. **54**(8): p. 6507-6522.
199. Rajasekar, N., et al., *Intranasal insulin improves cerebral blood flow, Nrf-2 expression and BDNF in STZ (ICV)-induced memory impaired rats.* Life Sci, 2017. **173**: p. 1-10.
200. Subramanian, S. and M. John, *Intranasal administration of insulin lowers amyloid-beta levels in rat model of diabetes.* Indian J Exp Biol, 2012. **50**(1): p. 41-4.
201. Sukhov, I.B., et al., *Long-term intranasal insulin administration improves spatial memory in male rats with prolonged type 1 diabetes mellitus and in healthy rats.* Dokl Biol Sci, 2013. **453**: p. 349-52.
202. Yang, Y., et al., *Intranasal insulin ameliorates tau hyperphosphorylation in a rat model of type 2 diabetes.* J Alzheimers Dis, 2013. **33**(2): p. 329-38.
203. Pancani, T., et al., *Imaging of a glucose analog, calcium and NADH in neurons and astrocytes: dynamic responses to depolarization and sensitivity to pioglitazone.* Cell Calcium, 2011. **50**(6): p. 548-58.
204. Maimaiti, S., et al., *Novel calcium-related targets of insulin in hippocampal neurons.* Neuroscience, 2017. **364**: p. 130-142.
205. Kumar, N.N., et al., *Relative vascular permeability and vascularity across different regions of the rat nasal mucosa: implications for nasal physiology and drug delivery.* Sci Rep, 2016. **6**: p. 31732.
206. Hanson, L.R. and W.H. Frey, 2nd, *Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease.* BMC Neurosci, 2008. **9 Suppl 3**: p. S5.
207. Benedict, C., et al., *Intranasal insulin improves memory in humans: superiority of insulin aspart.* Neuropsychopharmacology, 2007. **32**(1): p. 239-43.
208. Dash, S., et al., *Intranasal insulin suppresses endogenous glucose production in humans compared to placebo, in the presence of similar venous insulin concentration.* Diabetes, 2014.
209. Heni, M., et al., *Central insulin administration improves whole-body insulin sensitivity via hypothalamus and parasympathetic outputs in men.* Diabetes, 2014. **63**(12): p. 4083-8.
210. Reger, M.A., et al., *Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memory-impaired older adults.* J Alzheimers Dis, 2008. **13**(3): p. 323-31.
211. Duarte, A.I., et al., *Brain insulin signalling, glucose metabolism and females' reproductive aging: A dangerous triad in Alzheimer's disease.* Neuropharmacology, 2018. **136**(Pt B): p. 223-242.

212. Grichisch, Y., et al., *Differential effects of intranasal insulin and caffeine on cerebral blood flow.* Hum Brain Mapp, 2012. **33**(2): p. 280-7.
213. de la Monte, S.M., *Type 3 diabetes is sporadic Alzheimers disease: mini-review.* Eur Neuropsychopharmacol, 2014. **24**(12): p. 1954-60.
214. Schioth, H.B., et al., *Brain insulin signaling and Alzheimer's disease: current evidence and future directions.* Mol Neurobiol, 2012. **46**(1): p. 4-10.
215. Cashion, M.F., W.A. Banks, and A.J. Kastin, *Sequestration of centrally administered insulin by the brain: effects of starvation, aluminum, and TNF-alpha.* Horm Behav, 1996. **30**(3): p. 280-6.
216. Rhea, E.M., et al., *Intranasal Insulin Transport is Preserved in Aged SAMP8 Mice and is Altered by Albumin and Insulin Receptor Inhibition.* J Alzheimers Dis, 2017. **57**(1): p. 241-252.
217. Marks, D.R., et al., *Awake intranasal insulin delivery modifies protein complexes and alters memory, anxiety, and olfactory behaviors.* J Neurosci, 2009. **29**(20): p. 6734-51.
218. Zaia, A. and L. Piantanelli, *Insulin receptors in the brain cortex of aging mice.* Mech Ageing Dev, 2000. **113**(3): p. 227-32.
219. Pillion, D.J., M.D. Fyrberg, and E. Meezan, *Nasal absorption of mixtures of fast-acting and long-acting insulins.* Int J Pharm, 2010. **388**(1-2): p. 202-8.
220. Blalock, E.M., et al., *Effects of long-term pioglitazone treatment on peripheral and central markers of aging.* PLoS One, 2010. **5**(4): p. e10405.
221. Blalock, E.M., et al., *Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment.* J Neurosci, 2003. **23**(9): p. 3807-19.
222. Burger, C., et al., *Genome-wide analysis of aging and learning-related genes in the hippocampal dentate gyrus.* Neurobiol Learn Mem, 2008. **89**(4): p. 379-96.
223. Kadish, I., et al., *Hippocampal and cognitive aging across the lifespan: a bioenergetic shift precedes and increased cholesterol trafficking parallels memory impairment.* J Neurosci, 2009. **29**(6): p. 1805-16.
224. Loerch, P.M., et al., *Evolution of the aging brain transcriptome and synaptic regulation.* PLoS One, 2008. **3**(10): p. e3329.
225. Rowe, W.B., et al., *Hippocampal expression analyses reveal selective association of immediate-early, neuroenergetic, and myelinogenic pathways with cognitive impairment in aged rats.* J Neurosci, 2007. **27**(12): p. 3098-110.
226. Verbitsky, M., et al., *Altered hippocampal transcript profile accompanies an age-related spatial memory deficit in mice.* Learn Mem, 2004. **11**(3): p. 253-60.
227. Hargis, K.E. and E.M. Blalock, *Transcriptional signatures of brain aging and Alzheimer's disease: What are our rodent models telling us?* Behav Brain Res, 2017. **322**(Pt B): p. 311-328.
228. Zhang, Y., et al., *An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex.* J Neurosci, 2014. **34**(36): p. 11929-47.
229. Lindholm, A. and L.V. Jacobsen, *Clinical pharmacokinetics and pharmacodynamics of insulin aspart.* Clin Pharmacokinet, 2001. **40**(9): p. 641-59.
230. Vaidyanathan, B. and P.S. Menon, *Insulin analogues and management of diabetes mellitus.* Indian J Pediatr, 2000. **67**(6): p. 435-41.

231. Simpson, K.L. and C.M. Spencer, *Insulin aspart.* Drugs, 1999. **57**(5): p. 759-65; discussion 766-7.
232. Duckworth, W.C., *Insulin degradation: mechanisms, products, and significance.* Endocr Rev, 1988. **9**(3): p. 319-45.
233. Hill, J.M., et al., *Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas.* Neuroscience, 1986. **17**(4): p. 1127-38.
234. Fadool, D.A., et al., *Brain insulin receptor causes activity-dependent current suppression in the olfactory bulb through multiple phosphorylation of Kv1.3.* J Neurophysiol, 2000. **83**(4): p. 2332-48.
235. Lioutas, V.A. and V. Novak, *Intranasal insulin neuroprotection in ischemic stroke.* Neural Regen Res, 2016. **11**(3): p. 400-1.
236. Chiu, S.L., C.M. Chen, and H.T. Cline, *Insulin receptor signaling regulates synapse number, dendritic plasticity, and circuit function in vivo.* Neuron, 2008. **58**(5): p. 708-19.
237. Moasser, M.M., *The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis.* Oncogene, 2007. **26**(45): p. 6469-87.
238. Simons, T.J., *Calcium and neuronal function.* Neurosurg Rev, 1988. **11**(2): p. 119-29.
239. Catterall, W.A., *Structure and function of voltage-gated ion channels.* Annu Rev Biochem, 1995. **64**: p. 493-531.
240. Ophoff, R.A., et al., *Genetics and pathology of voltage-gated Ca2+ channels.* Histol Histopathol, 1998. **13**(3): p. 827-36.
241. Simms, B.A. and G.W. Zamponi, *Neuronal voltage-gated calcium channels: structure, function, and dysfunction.* Neuron, 2014. **82**(1): p. 24-45.
242. Schampel, A. and S. Kuerten, *Danger: High Voltage-The Role of Voltage-Gated Calcium Channels in Central Nervous System Pathology.* Cells, 2017. **6**(4).
243. Peterson, B.Z., et al., *Calmodulin is the Ca2+ sensor for Ca2+ -dependent inactivation of L-type calcium channels.* Neuron, 1999. **22**(3): p. 549-58.
244. Lisman, J., H. Schulman, and H. Cline, *The molecular basis of CaMKII function in synaptic and behavioural memory.* Nat Rev Neurosci, 2002. **3**(3): p. 175-90.
245. Bortolotto, Z.A. and G.L. Collingridge, *Involvement of calcium/calmodulin-dependent protein kinases in the setting of a molecular switch involved in hippocampal LTP.* Neuropharmacology, 1998. **37**(4-5): p. 535-44.
246. Cammarota, M., et al., *Learning-specific, time-dependent increases in hippocampal Ca2+/calmodulin-dependent protein kinase II activity and AMPA GluR1 subunit immunoreactivity.* Eur J Neurosci, 1998. **10**(8): p. 2669-76.
247. Hrabetova, S. and T.C. Sacktor, *Bidirectional regulation of protein kinase M zeta in the maintenance of long-term potentiation and long-term depression.* J Neurosci, 1996. **16**(17): p. 5324-33.
248. Verkhratsky, A., P.W. Landfield, and O. Thibault, *Preface. Ca2+ and neuronal pathology.* Eur J Pharmacol, 2002. **447**(2-3): p. 115-7.
249. Bruno, A.M., et al., *Altered ryanodine receptor expression in mild cognitive impairment and Alzheimer's disease.* Neurobiol Aging, 2012. **33**(5): p. 1001 e1-6.
250. Clodfelter, G.V., et al., *Sustained Ca2+-induced Ca2+-release underlies the post-glutamate lethal Ca2+ plateau in older cultured hippocampal neurons.* Eur J Pharmacol, 2002. **447**(2-3): p. 189-200.

251. Kruglikov, I., et al., *Diabetes-induced abnormalities in ER calcium mobilization in primary and secondary nociceptive neurons.* Pflugers Arch, 2004. **448**(4): p. 395-401.
252. Kumar, A. and T.C. Foster, *Enhanced long-term potentiation during aging is masked by processes involving intracellular calcium stores.* J Neurophysiol, 2004. **91**(6): p. 2437-44.
253. Landfield, P.W. and T.A. Pitler, *Prolonged Ca2+-dependent afterhyperpolarizations in hippocampal neurons of aged rats.* Science, 1984. **226**(4678): p. 1089-92.
254. Barrett, E.F. and J.N. Barret, *Separation of two voltage-sensitive potassium currents, and demonstration of a tetrodotoxin-resistant calcium current in frog motoneurones.* J Physiol, 1976. **255**(3): p. 737-74.
255. Hotson, J.R. and D.A. Prince, *A calcium-activated hyperpolarization follows repetitive firing in hippocampal neurons.* J Neurophysiol, 1980. **43**(2): p. 409-19.
256. Khachaturian, Z.S., *The role of calcium regulation in brain aging: reexamination of a hypothesis.* Aging (Milano), 1989. **1**(1): p. 17-34.
257. Landfield, P.W., *'Increased calcium-current' hypothesis of brain aging.* Neurobiol Aging, 1987. **8**(4): p. 346-7.
258. Biessels, G. and W.H. Gispen, *The calcium hypothesis of brain aging and neurodegenerative disorders: significance in diabetic neuropathy.* Life Sci, 1996. **59**(5-6): p. 379-87.
259. Landfield, P.W., *Increased hippocampal Ca2+ channel activity in brain aging and dementia. Hormonal and pharmacologic modulation.* Ann N Y Acad Sci, 1994. **747**: p. 351-64.
260. Landfield, P.W., *Aging-related increase in hippocampal calcium channels.* Life Sci, 1996. **59**(5-6): p. 399-404.
261. Thibault, O. and P.W. Landfield, *Increase in single L-type calcium channels in hippocampal neurons during aging.* Science, 1996. **272**(5264): p. 1017-20.
262. Thibault, O., et al., *Single-channel and whole-cell studies of calcium currents in young and aged rat hippocampal slice neurons.* J Neurosci Methods, 1995. **59**(1): p. 77-83.
263. Thibault, O., R. Hadley, and P.W. Landfield, *Elevated postsynaptic [Ca2+]i and L-type calcium channel activity in aged hippocampal neurons: relationship to impaired synaptic plasticity.* J Neurosci, 2001. **21**(24): p. 9744-56.
264. Michaelis, M.L., K. Johe, and T.E. Kitos, *Age-dependent alterations in synaptic membrane systems for Ca2+ regulation.* Mech Ageing Dev, 1984. **25**(1-2): p. 215-25.
265. Gibson, G., P. Perrino, and G.A. Dienel, *In vivo brain calcium homeostasis during aging.* Mech Ageing Dev, 1986. **37**(1): p. 1-12.
266. Disterhoft, J.F., et al., *Calcium-dependent afterhyperpolarization and learning in young and aging hippocampus.* Life Sci, 1996. **59**(5-6): p. 413-20.
267. Landfield, P.W., et al., *Mechanisms of neuronal death in brain aging and Alzheimer's disease: role of endocrine-mediated calcium dyshomeostasis.* J Neurobiol, 1992. **23**(9): p. 1247-60.
268. Thibault, O., et al., *Calcium dysregulation in neuronal aging and Alzheimer's disease: history and new directions.* Cell Calcium, 1998. **24**(5-6): p. 417-33.

269. Foster, T.C. and A. Kumar, *Calcium dysregulation in the aging brain.* Neuroscientist, 2002. **8**(4): p. 297-301.
270. Frazier, H.N., et al., *Calcium's role as nuanced modulator of cellular physiology in the brain.* Biochem Biophys Res Commun, 2017. **483**(4): p. 981-987.
271. Thibault, O., et al., *Hippocampal calcium dysregulation at the nexus of diabetes and brain aging.* Eur J Pharmacol, 2013. **719**(1-3): p. 34-43.
272. Thibault, O., et al., *Reduction in neuronal L-type calcium channel activity in a double knock-in mouse model of Alzheimer's disease.* Biochim Biophys Acta, 2012. **1822**(4): p. 546-9.
273. Kumar, A. and T.C. Foster, *Intracellular calcium stores contribute to increased susceptibility to LTD induction during aging.* Brain Res, 2005. **1031**(1): p. 125-8.
274. Ryan, M.M., et al., *Aging alters long-term potentiation--related gene networks and impairs synaptic protein synthesis in the rat hippocampus.* Neurobiol Aging, 2015. **36**(5): p. 1868-80.
275. Porter, N.M., et al., *Calcium channel density and hippocampal cell death with age in long-term culture.* J Neurosci, 1997. **17**(14): p. 5629-39.
276. Gant, J.C., et al., *FK506-binding protein 1b/12.6: a key to aging-related hippocampal Ca2+ dysregulation?* Eur J Pharmacol, 2014. **739**: p. 74-82.
277. Gant, J.C., et al., *Reversal of Aging-Related Neuronal Ca2+ Dysregulation and Cognitive Impairment by Delivery of a Transgene Encoding FK506-Binding Protein 12.6/1b to the Hippocampus.* J Neurosci, 2015. **35**(30): p. 10878-87.
278. Wu, W.W., M.M. Oh, and J.F. Disterhoft, *Age-related biophysical alterations of hippocampal pyramidal neurons: implications for learning and memory.* Ageing Res Rev, 2002. **1**(2): p. 181-207.
279. Huang, T.J., et al., *Diabetes-induced alterations in calcium homeostasis in sensory neurones of streptozotocin-diabetic rats are restricted to lumbar ganglia and are prevented by neurotrophin-3.* Diabetologia, 2002. **45**(4): p. 560-70.
280. Kamal, A., et al., *Increased spike broadening and slow afterhyperpolarization in CA1 pyramidal cells of streptozotocin-induced diabetic rats.* Neuroscience, 2003. **118**(2): p. 577-83.
281. Maimaiti, S., et al., *Short-lived diabetes in the young-adult ZDF rat does not exacerbate neuronal Ca(2+) biomarkers of aging.* Brain Res, 2015. **1621**: p. 214-21.
282. Chik, C.L., et al., *Insulin and insulin-like growth factor-I inhibit the L-type calcium channel current in rat pinealocytes.* Endocrinology, 1997. **138**(5): p. 2033-42.
283. Stella, S.L., Jr., E.J. Bryson, and W.B. Thoreson, *Insulin inhibits voltage-dependent calcium influx into rod photoreceptors.* Neuroreport, 2001. **12**(5): p. 947-51.
284. O'Malley, D., L.J. Shanley, and J. Harvey, *Insulin inhibits rat hippocampal neurones via activation of ATP-sensitive K+ and large conductance Ca2+-activated K+ channels.* Neuropharmacology, 2003. **44**(7): p. 855-63.
285. Huang, C.C., C.C. Lee, and K.S. Hsu, *An investigation into signal transduction mechanisms involved in insulin-induced long-term depression in the CA1 region of the hippocampus.* J Neurochem, 2004. **89**(1): p. 217-31.
286. Xing, C., et al., *Effects of insulin-like growth factor 1 on voltage-gated ion channels in cultured rat hippocampal neurons.* Brain Res, 2006. **1072**(1): p. 30-5.

287. Lebwohl, D.E., et al., *Expression of inducible membrane-anchored insulin receptor kinase enhances deoxyglucose uptake.* J Biol Chem, 1991. **266**(1): p. 386-90.
288. Lin, J.W., et al., *Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization.* Nat Neurosci, 2000. **3**(12): p. 1282-90.
289. Man, H.Y., et al., *Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization.* Neuron, 2000. **25**(3): p. 649-62.
290. Skeberdis, V.A., et al., *mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C.* Neuropharmacology, 2001. **40**(7): p. 856-65.
291. Vetiska, S.M., et al., *GABAA receptor-associated phosphoinositide 3-kinase is required for insulin-induced recruitment of postsynaptic GABAA receptors.* Neuropharmacology, 2007. **52**(1): p. 146-55.
292. Wan, Q., et al., *Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin.* Nature, 1997. **388**(6643): p. 686-90.
293. McNay, E.C., L.A. Sandusky, and J. Pearson-Leary, *Hippocampal insulin microinjection and in vivo microdialysis during spatial memory testing.* J Vis Exp, 2013(71): p. e4451.
294. Stranahan, A.M., *Models and mechanisms for hippocampal dysfunction in obesity and diabetes.* Neuroscience, 2015. **309**: p. 125-39.
295. Anderson, K.L., et al., *Impact of Single or Repeated Dose Intranasal Zinc-free Insulin in Young and Aged F344 Rats on Cognition, Signaling, and Brain Metabolism.* J Gerontol A Biol Sci Med Sci, 2016.
296. Brini, M. and E. Carafoli, *Calcium pumps in health and disease.* Physiol Rev, 2009. **89**(4): p. 1341-78.
297. Clapham, D.E., *Calcium signaling.* Cell, 2007. **131**(6): p. 1047-58.
298. Jiang, L., et al., *Decreases in plasma membrane Ca(2)(+)-ATPase in brain synaptic membrane rafts from aged rats.* J Neurochem, 2012. **123**(5): p. 689-99.
299. Michaelis, E.K., et al., *High affinity Ca2+-stimulated Mg2+-dependent ATPase in rat brain synaptosomes, synaptic membranes, and microsomes.* J Biol Chem, 1983. **258**(10): p. 6101-8.
300. Michaelis, M.L. and E.K. Michaelis, *Ca++ fluxes in resealed synaptic plasma membrane vesicles.* Life Sci, 1981. **28**(1): p. 37-45.
301. Schmidt, N., et al., *Neuroplastin and Basigin Are Essential Auxiliary Subunits of Plasma Membrane Ca2+-ATPases and Key Regulators of Ca2+ Clearance.* Neuron, 2017. **96**(4): p. 827-838 e9.
302. Wang, X. and E.K. Michaelis, *Selective neuronal vulnerability to oxidative stress in the brain.* Front Aging Neurosci, 2010. **2**: p. 12.
303. Gant, J.C., et al., *Early and simultaneous emergence of multiple hippocampal biomarkers of aging is mediated by Ca2+-induced Ca2+ release.* J Neurosci, 2006. **26**(13): p. 3482-90.
304. Murchison, D., et al., *Enhanced calcium buffering in F344 rat cholinergic basal forebrain neurons is associated with age-related cognitive impairment.* J Neurophysiol, 2009. **102**(4): p. 2194-207.

305. Wilkins, H.M., et al., *Oxaloacetate activates brain mitochondrial biogenesis, enhances the insulin pathway, reduces inflammation and stimulates neurogenesis.* Hum Mol Genet, 2014. **23**(24): p. 6528-41.
306. Pancani, T., et al., *Distinct modulation of voltage-gated and ligand-gated Ca2+ currents by PPAR-gamma agonists in cultured hippocampal neurons.* J Neurochem, 2009. **109**(6): p. 1800-11.
307. Furler, S., et al., *Recombinant AAV vectors containing the foot and mouth disease virus 2A sequence confer efficient bicistronic gene expression in cultured cells and rat substantia nigra neurons.* Gene Ther, 2001. **8**(11): p. 864-73.
308. Mizuguchi, H., et al., *IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector.* Mol Ther, 2000. **1**(4): p. 376-82.
309. Bodhinathan, K., A. Kumar, and T.C. Foster, *Redox sensitive calcium stores underlie enhanced after hyperpolarization of aged neurons: role for ryanodine receptor mediated calcium signaling.* J Neurophysiol, 2010. **104**(5): p. 2586-93.
310. Norris, C.M., S. Halpain, and T.C. Foster, *Reversal of age-related alterations in synaptic plasticity by blockade of L-type Ca2+ channels.* J Neurosci, 1998. **18**(9): p. 3171-9.
311. Thibault, O., N.M. Porter, and P.W. Landfield, *Low Ba2+ and Ca2+ induce a sustained high probability of repolarization openings of L-type Ca2+ channels in hippocampal neurons: physiological implications.* Proc Natl Acad Sci U S A, 1993. **90**(24): p. 11792-6.
312. Sonne, O. and I.A. Simpson, *Internalization of insulin and its receptor in the isolated rat adipose cell. Time-course and insulin concentration dependency.* Biochim Biophys Acta, 1984. **804**(4): p. 404-13.
313. Standaert, M.L. and R.J. Pollet, *Equilibrium model for insulin-induced receptor down-regulation. Regulation of insulin receptors in differentiated BC3H-1 myocytes.* J Biol Chem, 1984. **259**(4): p. 2346-54.
314. Wang, C.C., et al., *Insulin-induced internalization of the insulin receptor in the isolated rat adipose cell. Detection of the internalized 138-kilodalton receptor subunit using a photoaffinity 125I-insulin.* J Biol Chem, 1983. **258**(8): p. 5129-34.
315. Lee, C.C., et al., *Insulin stimulates postsynaptic density-95 protein translation via the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway.* J Biol Chem, 2005. **280**(18): p. 18543-50.
316. Kim, E.Y. and S.E. Dryer, *Effects of insulin and high glucose on mobilization of slo1 BKCa channels in podocytes.* J Cell Physiol, 2011. **226**(9): p. 2307-15.
317. Benomar, Y., et al., *Insulin and leptin induce Glut4 plasma membrane translocation and glucose uptake in a human neuronal cell line by a phosphatidylinositol 3-kinase- dependent mechanism.* Endocrinology, 2006. **147**(5): p. 2550-6.
318. Biessels, G.J. and L.P. Reagan, *Hippocampal insulin resistance and cognitive dysfunction.* Nat Rev Neurosci, 2015. **16**(11): p. 660-71.
319. Grillo, C.A., et al., *Hippocampal Insulin Resistance Impairs Spatial Learning and Synaptic Plasticity.* Diabetes, 2015. **64**(11): p. 3927-36.
320. McNay, E.C., et al., *Hippocampal memory processes are modulated by insulin and high-fat-induced insulin resistance.* Neurobiol Learn Mem, 2010. **93**(4): p. 546-53.

321. Zhao, W.Q. and D.L. Alkon, *Role of insulin and insulin receptor in learning and memory.* Mol Cell Endocrinol, 2001. **177**(1-2): p. 125-34.
322. Michaelis, M.L., L. Jiang, and E.K. Michaelis, *Isolation of Synaptosomes, Synaptic Plasma Membranes, and Synaptic Junctional Complexes.* Methods Mol Biol, 2017. **1538**: p. 107-119.
323. Clarke, D.D. and L. Sokoloff, *Regulation of Cerebral Metabolic Rate*, in *Basic Neurochemistry: Molecular, Cellular and Medical Aspects*, G.J. Siegel, et al., Editors. 1999, Lippincott-Raven: Philadelphia, PA, USA.
324. Szablewski, L., *Glucose Transporters in Brain: In Health and in Alzheimer's Disease.* J Alzheimers Dis, 2016.
325. Oka, Y., et al., *C-terminal truncated glucose transporter is locked into an inward-facing form without transport activity.* Nature, 1990. **345**(6275): p. 550-3.
326. Fox, P.T., et al., *Nonoxidative glucose consumption during focal physiologic neural activity.* Science, 1988. **241**(4864): p. 462-4.
327. Magistretti, P.J., *Neuron-glia metabolic coupling and plasticity.* J Exp Biol, 2006. **209**(Pt 12): p. 2304-11.
328. Mueckler, M.M., *The molecular biology of mammalian glucose transporters.* Curr Opin Nephrol Hypertens, 1992. **1**(1): p. 12-20.
329. Bell, G.I., et al., *Molecular biology of mammalian glucose transporters.* Diabetes Care, 1990. **13**(3): p. 198-208.
330. Kasanicki, M.A. and P.F. Pilch, *Regulation of glucose-transporter function.* Diabetes Care, 1990. **13**(3): p. 219-27.
331. Shah, K., S. Desilva, and T. Abbruscato, *The role of glucose transporters in brain disease: diabetes and Alzheimer's Disease.* Int J Mol Sci, 2012. **13**(10): p. 12629-55.
332. Fladeby, C., R. Skar, and G. Serck-Hanssen, *Distinct regulation of glucose transport and GLUT1/GLUT3 transporters by glucose deprivation and IGF-I in chromaffin cells.* Biochim Biophys Acta, 2003. **1593**(2-3): p. 201-8.
333. Zheng, Q., et al., *Glucose regulation of glucose transporters in cultured adult and fetal hepatocytes.* Metabolism, 1995. **44**(12): p. 1553-8.
334. Baldwin, S.A., L.F. Barros, and M. Griffiths, *Trafficking of glucose transporters--signals and mechanisms.* Biosci Rep, 1995. **15**(6): p. 419-26.
335. Boado, R.J., D. Wu, and M. Windisch, *In vivo upregulation of the blood-brain barrier GLUT1 glucose transporter by brain-derived peptides.* Neurosci Res, 1999. **34**(4): p. 217-24.
336. Jurcovicova, J., *Glucose transport in brain - effect of inflammation.* Endocr Regul, 2014. **48**(1): p. 35-48.
337. Wilson, C.M., et al., *Regulation of cell surface GLUT1, GLUT3, and GLUT4 by insulin and IGF-I in L6 myotubes.* FEBS Lett, 1995. **368**(1): p. 19-22.
338. Bruckner, B.A., et al., *Regulation of brain glucose transporters by glucose and oxygen deprivation.* Metabolism, 1999. **48**(4): p. 422-31.
339. Ouiddir, A., et al., *Hypoxia upregulates activity and expression of the glucose transporter GLUT1 in alveolar epithelial cells.* Am J Respir Cell Mol Biol, 1999. **21**(6): p. 710-8.

340. Taha, C., et al., *The insulin-dependent biosynthesis of GLUT1 and GLUT3 glucose transporters in L6 muscle cells is mediated by distinct pathways. Roles of p21ras and pp70 S6 kinase.* J Biol Chem, 1995. **270**(42): p. 24678-81.
341. Wieman, H.L., J.A. Wofford, and J.C. Rathmell, *Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking.* Mol Biol Cell, 2007. **18**(4): p. 1437-46.
342. Maher, F., et al., *Expression of two glucose transporters, GLUT1 and GLUT3, in cultured cerebellar neurons: Evidence for neuron-specific expression of GLUT3.* Mol Cell Neurosci, 1991. **2**(4): p. 351-60.
343. Maher, F. and I.A. Simpson, *The GLUT3 glucose transporter is the predominant isoform in primary cultured neurons: assessment by biosynthetic and photoaffinity labelling.* Biochem J, 1994. **301 (Pt 2)**: p. 379-84.
344. Haber, R.S., et al., *Tissue distribution of the human GLUT3 glucose transporter.* Endocrinology, 1993. **132**(6): p. 2538-43.
345. Maher, F., et al., *Expression of mouse-GLUT3 and human-GLUT3 glucose transporter proteins in brain.* Biochem Biophys Res Commun, 1992. **182**(2): p. 703-11.
346. Yano, H., et al., *Tissue distribution and species difference of the brain type glucose transporter (GLUT3).* Biochem Biophys Res Commun, 1991. **174**(2): p. 470-7.
347. Nagamatsu, S., et al., *Neuron-specific glucose transporter (NSGT): CNS distribution of GLUT3 rat glucose transporter (RGT3) in rat central neurons.* FEBS Lett, 1993. **334**(3): p. 289-95.
348. Maher, F., *Immunolocalization of GLUT1 and GLUT3 glucose transporters in primary cultured neurons and glia.* J Neurosci Res, 1995. **42**(4): p. 459-69.
349. Mantych, G.J., et al., *Cellular localization and characterization of Glut 3 glucose transporter isoform in human brain.* Endocrinology, 1992. **131**(3): p. 1270-8.
350. McCall, A.L., et al., *Immunohistochemical localization of the neuron-specific glucose transporter (GLUT3) to neuropil in adult rat brain.* Brain Res, 1994. **659**(1-2): p. 292-7.
351. McEwen, B.S. and L.P. Reagan, *Glucose transporter expression in the central nervous system: relationship to synaptic function.* Eur J Pharmacol, 2004. **490**(1-3): p. 13-24.
352. Nagamatsu, S., et al., *Gene expression of GLUT3 glucose transporter regulated by glucose in vivo in mouse brain and in vitro in neuronal cell cultures from rat embryos.* Biochem J, 1994. **300 (Pt 1)**: p. 125-31.
353. Yu, J., et al., *IGF-1 induces hypoxia-inducible factor 1alpha-mediated GLUT3 expression through PI3K/Akt/mTOR dependent pathways in PC12 cells.* Brain Res, 2012. **1430**: p. 18-24.
354. Cidad, P., A. Almeida, and J.P. Bolanos, *Inhibition of mitochondrial respiration by nitric oxide rapidly stimulates cytoprotective GLUT3-mediated glucose uptake through 5'-AMP-activated protein kinase.* Biochem J, 2004. **384**(Pt 3): p. 629-36.
355. Dimitriadis, G., et al., *Thyroid hormone excess increases basal and insulin-stimulated recruitment of GLUT3 glucose transporters on cell surface.* Horm Metab Res, 2005. **37**(1): p. 15-20.

356. Reagan, L.P., et al., *Oxidative stress and HNE conjugation of GLUT3 are increased in the hippocampus of diabetic rats subjected to stress.* Brain Res, 2000. **862**(1-2): p. 292-300.
357. Ximenes da Silva, A., et al., *Glucose transport and utilization are altered in the brain of rats deficient in n-3 polyunsaturated fatty acids.* J Neurochem, 2002. **81**(6): p. 1328-37.
358. Ferreira, J.M., A.L. Burnett, and G.A. Rameau, *Activity-dependent regulation of surface glucose transporter-3.* J Neurosci, 2011. **31**(6): p. 1991-9.
359. Rajakumar, A., et al., *Trans-activators regulating neuronal glucose transporter isoform-3 gene expression in mammalian neurons.* J Biol Chem, 2004. **279**(25): p. 26768-79.
360. Bilan, P.J., et al., *Detection of the GLUT3 facilitative glucose transporter in rat L6 muscle cells: regulation by cellular differentiation, insulin and insulin-like growth factor-I.* Biochem Biophys Res Commun, 1992. **186**(2): p. 1129-37.
361. Dimitriadis, G., et al., *Evaluation of glucose transport and its regulation by insulin in human monocytes using flow cytometry.* Cytometry A, 2005. **64**(1): p. 27-33.
362. Liu, C.C., et al., *Neuronal LRP1 regulates glucose metabolism and insulin signaling in the brain.* J Neurosci, 2015. **35**(14): p. 5851-9.
363. Uemura, E. and H.W. Greenlee, *Insulin regulates neuronal glucose uptake by promoting translocation of glucose transporter GLUT3.* Exp Neurol, 2006. **198**(1): p. 48-53.
364. An, Y., et al., *Evidence for brain glucose dysregulation in Alzheimer's disease.* Alzheimers Dement, 2018. **14**(3): p. 318-329.
365. Fattoretti, P., et al., *Quantitative immunohistochemistry of glucose transport protein (Glut3) expression in the rat hippocampus during aging.* J Histochem Cytochem, 2001. **49**(5): p. 671-2.
366. Vannucci, S.J., E.M. Gibbs, and I.A. Simpson, *Glucose utilization and glucose transporter proteins GLUT-1 and GLUT-3 in brains of diabetic (db/db) mice.* Am J Physiol, 1997. **272**(2 Pt 1): p. E267-74.
367. Oliveira, L.T., et al., *Exogenous beta-amyloid peptide interferes with GLUT4 localization in neurons.* Brain Res, 2015. **1615**: p. 42-50.
368. Pearson-Leary, J. and E.C. McNay, *Novel Roles for the Insulin-Regulated Glucose Transporter-4 in Hippocampally Dependent Memory.* J Neurosci, 2016. **36**(47): p. 11851-11864.
369. Bauer, J., et al., *Causes and consequences of neuronal energy deficit in sporadic Alzheimer's disease.* Ann N Y Acad Sci, 1997. **826**: p. 379-81.
370. Matioli, M. and R. Nitrini, *Mechanisms linking brain insulin resistance to Alzheimer's disease.* Dement Neuropsychol, 2015. **9**(2): p. 96-102.
371. Mosconi, L., *Brain glucose metabolism in the early and specific diagnosis of Alzheimer's disease. FDG-PET studies in MCI and AD.* Eur J Nucl Med Mol Imaging, 2005. **32**(4): p. 486-510.
372. Mosconi, L., et al., *Metabolic interaction between ApoE genotype and onset age in Alzheimer's disease: implications for brain reserve.* J Neurol Neurosurg Psychiatry, 2005. **76**(1): p. 15-23.
373. Mosconi, L., et al., *Reduced hippocampal metabolism in MCI and AD: automated FDG-PET image analysis.* Neurology, 2005. **64**(11): p. 1860-7.

374. Mosconi, L., et al., *Hippocampal hypometabolism predicts cognitive decline from normal aging.* Neurobiol Aging, 2008. **29**(5): p. 676-92.
375. Mosconi, L., A. Pupi, and M.J. De Leon, *Brain glucose hypometabolism and oxidative stress in preclinical Alzheimer's disease.* Ann N Y Acad Sci, 2008. **1147**: p. 180-95.
376. Macklin, L., et al., *Glucose tolerance and insulin sensitivity are impaired in APP/PS1 transgenic mice prior to amyloid plaque pathogenesis and cognitive decline.* Exp Gerontol, 2017. **88**: p. 9-18.
377. Pedros, I., et al., *Early alterations in energy metabolism in the hippocampus of APPswe/PS1dE9 mouse model of Alzheimer's disease.* Biochim Biophys Acta, 2014. **1842**(9): p. 1556-66.
378. Dietrich, W.D., et al., *Widespread metabolic depression and reduced somatosensory circuit activation following traumatic brain injury in rats.* J Neurotrauma, 1994. **11**(6): p. 629-40.
379. Garcia-Panach, J., et al., *A voxel-based analysis of FDG-PET in traumatic brain injury: regional metabolism and relationship between the thalamus and cortical areas.* J Neurotrauma, 2011. **28**(9): p. 1707-17.
380. Gonzalez-Reyes, R.E., et al., *Alterations in Glucose Metabolism on Cognition: A Possible Link Between Diabetes and Dementia.* Curr Pharm Des, 2016. **22**(7): p. 812-8.
381. Gross, H., et al., *Local cerebral glucose metabolism in patients with long-term behavioral and cognitive deficits following mild traumatic brain injury.* J Neuropsychiatry Clin Neurosci, 1996. **8**(3): p. 324-34.
382. Soustiel, J.F., et al., *Monitoring of cerebral blood flow and metabolism in traumatic brain injury.* J Neurotrauma, 2005. **22**(9): p. 955-65.
383. Goyal, M.S., et al., *Loss of Brain Aerobic Glycolysis in Normal Human Aging.* Cell Metab, 2017. **26**(2): p. 353-360 e3.
384. Grillo, C.A., et al., *Insulin resistance and hippocampal dysfunction: Disentangling peripheral and brain causes from consequences.* Exp Neurol, 2019.
385. Zhang, Y.L. and L. Wang, *[Relationships of glucose transporter 4 with cognitive changes induced by high fat diet and glucose metabolism in hippocampus].* Sheng Li Xue Bao, 2016. **68**(3): p. 335-42.
386. Garcia-Casares, N., et al., *Structural and functional brain changes in middle-aged type 2 diabetic patients: a cross-sectional study.* J Alzheimers Dis, 2014. **40**(2): p. 375-86.
387. Vannucci, S.J., et al., *GLUT4 glucose transporter expression in rodent brain: effect of diabetes.* Brain Res, 1998. **797**(1): p. 1-11.
388. Cholerton, B., L.D. Baker, and S. Craft, *Insulin resistance and pathological brain ageing.* Diabet Med, 2011. **28**(12): p. 1463-75.
389. Schwartz, M.W., et al., *Insulin binding to brain capillaries is reduced in genetically obese, hyperinsulinemic Zucker rats.* Peptides, 1990. **11**(3): p. 467-72.
390. Kullmann, S., et al., *Hypothalamic insulin responsiveness is associated with pancreatic insulin secretion in humans.* Physiol Behav, 2017. **176**: p. 134-138.
391. Cong, L.N., et al., *Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells.* Mol Endocrinol, 1997. **11**(13): p. 1881-90.

392. Kohn, A.D., et al., *Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation.* J Biol Chem, 1996. **271**(49): p. 31372-8.
393. Joost, H.G., *Structural and functional heterogeneity of insulin receptors.* Cell Signal, 1995. **7**(2): p. 85-91.
394. Vannucci, S.J., F. Maher, and I.A. Simpson, *Glucose transporter proteins in brain: delivery of glucose to neurons and glia.* Glia, 1997. **21**(1): p. 2-21.
395. Choeiri, C., W. Staines, and C. Messier, *Immunohistochemical localization and quantification of glucose transporters in the mouse brain.* Neuroscience, 2002. **111**(1): p. 19-34.
396. McNay, E.C. and P.E. Gold, *Age-related differences in hippocampal extracellular fluid glucose concentration during behavioral testing and following systemic glucose administration.* J Gerontol A Biol Sci Med Sci, 2001. **56**(2): p. B66-71.
397. Natarajan, A. and F. Srienc, *Dynamics of glucose uptake by single Escherichia coli cells.* Metab Eng, 1999. **1**(4): p. 320-33.
398. Brown, A.M. and B.R. Ransom, *Astrocyte glycogen and brain energy metabolism.* Glia, 2007. **55**(12): p. 1263-71.
399. DiNuzzo, M., et al., *Glycogenolysis in astrocytes supports blood-borne glucose channeling not glycogen-derived lactate shuttling to neurons: evidence from mathematical modeling.* J Cereb Blood Flow Metab, 2010. **30**(12): p. 1895-904.
400. Barkhoudarian, G., D.A. Hovda, and C.C. Giza, *The molecular pathophysiology of concussive brain injury.* Clin Sports Med, 2011. **30**(1): p. 33-48, vii-iii.
401. Bingham, E.M., et al., *The role of insulin in human brain glucose metabolism: an 18fluoro-deoxyglucose positron emission tomography study.* Diabetes, 2002. **51**(12): p. 3384-90.
402. Li, Y., et al., *Regional analysis of FDG and PIB-PET images in normal aging, mild cognitive impairment, and Alzheimer's disease.* Eur J Nucl Med Mol Imaging, 2008. **35**(12): p. 2169-81.
403. Belanger, M., I. Allaman, and P.J. Magistretti, *Differential effects of pro- and anti-inflammatory cytokines alone or in combinations on the metabolic profile of astrocytes.* J Neurochem, 2011. **116**(4): p. 564-76.
404. Diaz-Garcia, C.M., et al., *Neuronal Stimulation Triggers Neuronal Glycolysis and Not Lactate Uptake.* Cell Metab, 2017. **26**(2): p. 361-374 e4.
405. Lundgaard, I., et al., *Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism.* Nat Commun, 2015. **6**: p. 6807.
406. Shin, B.C., et al., *Neural Deletion of Glucose Transporter Isoform 3 Creates Distinct Postnatal and Adult Neurobehavioral Phenotypes.* J Neurosci, 2018. **38**(44): p. 9579-9599.
407. Yu, S., et al., *Hypoxic preconditioning up-regulates glucose transport activity and glucose transporter (GLUT1 and GLUT3) gene expression after acute anoxic exposure in the cultured rat hippocampal neurons and astrocytes.* Brain Res, 2008. **1211**: p. 22-9.
408. Gomez, O., et al., *Developmental regulation of glucose transporters GLUT3, GLUT4 and GLUT8 in the mouse cerebellar cortex.* J Anat, 2010. **217**(5): p. 616-23.

409. Rolla, A., *Pharmacokinetic and pharmacodynamic advantages of insulin analogues and premixed insulin analogues over human insulins: impact on efficacy and safety.* Am J Med, 2008. **121**(6 Suppl): p. S9-S19.
410. Ma, J., et al., *Prolonged insulin stimulation down-regulates GLUT4 through oxidative stress-mediated retromer inhibition by a protein kinase CK2-dependent mechanism in 3T3-L1 adipocytes.* J Biol Chem, 2014. **289**(1): p. 133-42.
411. Asano, T., et al., *Characterization of GLUT3 protein expressed in Chinese hamster ovary cells.* Biochem J, 1992. **288 (Pt 1)**: p. 189-93.
412. Nishimura, H., et al., *Kinetics of GLUT1 and GLUT4 glucose transporters expressed in Xenopus oocytes.* J Biol Chem, 1993. **268**(12): p. 8514-20.
413. Palfreyman, R.W., et al., *Kinetic resolution of the separate GLUT1 and GLUT4 glucose transport activities in 3T3-L1 cells.* Biochem J, 1992. **284 (Pt 1)**: p. 275-82.

VITA

HILAREE N. FRAZIER

EDUCATION

M.S. General Biology 2016
Eastern Kentucky University

B.S. General Biology 2011
Eastern Kentucky University

RESEARCH EXPERIENCE

Dissertation Research 2014-2019
University of Kentucky
Director: Dr. Olivier Thibault, Department of Pharmacology & Nutritional Sciences

Graduate Thesis Research 2012-2014
Eastern Kentucky University
Advisor: Dr. Marcia Pierce, Department of Biological Sciences

Undergraduate Research 2010-2011
Eastern Kentucky University
Advisor: Dr. Marcia Pierce, Department of Biological Sciences

RELATED EXPERIENCE

Graduate Teaching Assistant (TA) 2013-2014
Eastern Kentucky University, Richmond, KY
Graduate Lab Assistant (GA) 2012-2013
Eastern Kentucky University, Richmond, KY

PUBLICATIONS

Frazier HN, Ghoweri AO, Anderson KL, Lin RL, Popa GJ, Mendenhall MD, Reagan LP, Craven RJ, Thibault O. Elevating Insulin Receptor Signaling Using a Constitutively Active Human Insulin Receptor Increases Glucose Metabolism and Expression of GLUT3 in Cultured Hippocampal Neurons. (*submitted)*

Porter NM, Brewer LD, Anderson KL, Hoffman J, Thibault JR, Gant JC, **Frazier HN**, Ghoweri AO, Kraner SD, Landfield P, Blalock EM, Thibault O. Sexually Dimorphic Effects of Dietary Vitamin D3 Supplementation on Cognition and the Gut Microbiome in Aging Rats (P14-006-19). *Curr Dev Nutr.* 2019;epub doi.org/10.1093/cdn/nzz052.P14-006-19

Frazier HN, Ghoweri AO, Sudkamp E, Johnson ES, Anderson KL, Fox G, Vatthanaphone K, Xia M, Lin RL, Hargis-Staggs KE, Porter NM, Pauly JR, Blalock EM, Thibault O. Long-Term Intranasal Insulin Aspart: A Profile of Gene Expression, Memory, and Insulin Receptors in Aged Fisher 344 Rats. *J Gerontol A Biol Sci Med Sci.* 2019;epub doi.org/10.1093/gerona/glz105

Frazier HN, Ghoweri AO, Anderson KL, Lin RL, Porter NM, Thibault O. Broadening the Definition of Brain Insulin Resistance in Aging and Alzheimer's Disease. *Experi Neurol.* 2019;313:79-87. doi.org/10.1016/j.expneurol.2018.12.007

Hampton KK, **Frazier HN**, Anderson KL, Thibault O, Craven RJ. Insulin Receptor Plasma Membrane Levels Increased by the Progesterone Receptor Membrane Component 1. *Mol Pharmacol.* 2018;94(1):665-673. doi.org/10.1124/mol.117.110510

Frazier HN, Anderson KL, Maimaiti S, Ghoweri AO, Kraner SD, Popa GJ, Hampton KK, Mendenhall MD, Norris CM, Craven RJ, Thibault O. Expression of a Constitutively Active Human Insulin Receptor in Hippocampal Neurons Does Not Alter VGCC Currents. *Neurochem Res*. 2019;44:269-280. doi.org/10.1007/s11064-018-2510-2

Maimaiti S, **Frazier HN**, Anderson KL, Ghoweri AO, Brewer LD, Porter NM, Thibault O. Novel Calcium-Related Targets of Insulin in Hippocampal Neurons. *Neuroscience.* 2017;364:130-142. doi.org/10.1016/j.neuroscience.2017.09

Frazier HN, Maimaiti S, Anderson KL, Brewer LD, Gant JC, Porter NM, Thibault O. Calcium's Role as Nuanced Modulator of Cellular Physiology in the Brain. *Biochem Biophys Res Commun*. 2017;483(4):981-987. doi.org/10.1016/j.bbrc.2016.08.105

Anderson KL, **Frazier HN**, Maimaiti S, Bakshi VV, Majeed ZR, Brewer LD, Porter LM, Lin AL, Thibault O. Impact of Single or Repeated Dose Intranasal Zinc-Free Insulin in Young and Aged F344 Rats on Cognition, Signaling, and Brain Metabolism. *J Gerontol A Biol Sci Med Sci*. 2017;72(2):189-197. doi.org10.1093/gerona/glw065

FELLOWSHIPS AND AWARDS

1st Place Winner Presentation at the Barnstable Brown Obesity & Diabetes Research Day Poster Session, *Barnstable Brown Diabetes Center, University of Kentucky Healthcare* **2019**

Recipient of the Dept. of Pharmacology & Nutritional Sciences Student of the Year Award, *Dept. of Pharmacology & Nutritional Sciences, University of Kentucky College of Medicine* **2019**

Recipient of the AAAS/Science Program for Excellence in Science, *American Association for the Advancement of Science* **2019**

Fellowship for Excellence in Graduate Research, *University of Kentucky College of Medicine* **2018-2019**

ISN 2018 Symposium Travel Award, *International Society for Neurogastronomy* **2018**

Pharmacology & Nutritional Sciences: Multidisciplinary Approaches for Metabolic Disease, *National Institutes of Health: NIDDK [T32DK007778]* **2016-2018**

Kentucky Bridge to a Biomedical Doctorate for Appalachian Students, *National Institutes of Health: NIGMS [R25GM102776]* **2012-2014**

Excellence Award Scholarship, *Eastern Kentucky University* **2007-2011**