Effects of GLT-1 loss on central nervous system insulin signaling and implications for Alzheimer's disease pathogenesis

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Abstract

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Regulation of glutamatergic neurotransmission in the central nervous system (CNS) by glutamate transporters allows for the fine balance between tightly regulated signaling and prevention of glutamate-induced neurotoxicity. In forebrain regions, glutamate is essential for cognition along with regulation of metabolism. The glutamate transporter GLT-1 (also known as EAAT2) is responsible for the majority of glutamate uptake in the forebrain and in keeping with its dominant role in maintaining glutamate homeostasis, loss or dysfunction of GLT-1 has been implicated in multiple CNS disorders, including Alzheimer's disease (AD). In AD, GLT-1 levels begin falling early and are reduced by as much as 50% in later stages of the disease. These findings, along with others, suggest a role of glutamate dyshomeostasis in AD pathogenesis.

In conjunction with glutamatergic disturbances in AD, there has been a wealth of recent evidence identifying insulin signaling disturbances in the brains of individuals with AD. The insulin signaling changes identified are believed to be indicative of a state of insulin resistance in the brain, which have been found primarily in forebrain regions such as the cortex and

hippocampus. Along with regulating metabolic changes in both the CNS and periphery, there is evidence that insulin action in the brain is critical for modulating cognitive processes. Similar to GLT-1 loss in AD, insulin signaling changes have been identified early in the course of the disease. Interestingly, GLT-1 expression levels have also been found to be regulated by insulin signaling. Taken collectively, these findings suggest that glutamatergic and insulin signaling share several similarities in the brain, particularly that both are important for metabolic and cognitive processes, which when disturbed may each play a role in AD pathogenesis. The goal of this thesis was to examine the relationship between GLT-1 loss and insulin signaling disturbances in the context of AD.

Previous work from the Cook laboratory has shown that partial GLT-1 loss (to levels consistent with those identified in AD cases) causes deficits in cognitive function in a mouse model of AD (Mookherjee et al., 2011). However, partial loss of GLT-1 resulted in only modest changes to amyloid processing in these mice suggesting that increased amyloid pathology was not responsible for the accelerated onset of cognitive deficits. Given the interactions between glutamatergic and insulin signaling and their similar functions in the brain, we examined if partial GLT-1 loss resulted in disturbances to CNS insulin signaling in these same animals. We found alterations to several components of the pathway, including decreased insulin receptor and IRS-1 activation along with increased Akt activation, indicative of an overall reduction in insulin signaling in the brain. These changes mirrored the onset of cognitive deficits previously identified in these mice and were similar to insulin signaling disturbances identified in AD brains.

As insulin signaling changes in AD have been identified predominantly in neurons and neuronal GLT-1 is responsible for a significant portion of glutamate uptake even though its

expression levels are low, we utilized primary cortical neurons to determine the mechanistic relationship between GLT-1 loss and insulin signaling alterations. Loss of neuronal GLT-1 function *in vitro* resulted in a significant decrease in insulin-evoked phosphorylation of the insulin receptor along with significant reductions in both the insulin-evoked and basal phosphorylation states of other insulin signaling proteins including Akt, GSK-3β, and mTOR. Total IRS-1 levels were also found to be significantly reduced by loss of GLT-1 function. Insulin signaling changes induced by GLT-1 inhibition were reversed by scavenging of extracellular glutamate and inhibition of NMDA-type glutamate receptors.

Collectively, these results suggest that loss of GLT-1 led to dyshomeostasis of glutamatergic signaling thereby disturbing insulin signaling in the brain, which was accompanied by deficits in cognitive function. Furthermore, these changes occurred early, similar to their appearance in AD. Thus, this study links two previously distinct components of AD, which may together play a role in AD pathogenesis.

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LIST OF ABBREVIATIONS

Aβ	amyloid β
ACE	angiotensin-converting enzyme
AD	Alzheimer's disease
AgRP	Agouti-related protein
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	apolipoprotein E
APP	amyloid precursor protein
BBB	blood-brain barrier
CART	cocaine- and amphetamine-regulated transcript
CDC	Centers for Disease Control and Prevention
CNS	central nervous system
DHK	dihydrokainic acid
EAAT	excitatory amino acid transporter
ECE	endothelin-converting enzyme
GPCR	G protein-coupled receptor
GSK-3β	glycogen synthase kinase-3β
HD	Huntington's disease
ICV	intracerebroventricular
IDE	insulin-degrading enzyme
IGF	insulin-like growth factor
IR	insulin receptor
IRS	insulin receptor substrate
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
MMP	matrix metalloproteinase

mTOR	mammalian target of rapamycin
NIRKO	neuron-specific insulin receptor knockout
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
p70 S6K	p70 S6 kinase
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PKC	protein kinase C
POMC	proopiomelanocortin
PS1	presenilin 1
PS2	presenilin 2
PTB	phosphotyrosine-binding
SCA5	spinocerebellar ataxia type 5
SH2	Src homology 2
T2DM	type 2 diabetes mellitus
TBI	traumatic brain injury
TBOA	DL-threo-β-benzyloxyaspartate
TNFα	tumor necrosis factor α
VGLUT	vesicular glutamate transporter

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PREFACE

Data and text in Chapter 2 of this dissertation is reprinted, with some changes, from the following publication:

Journal of Alzheimer's Disease, Volume 45(2). Meeker KD, Meabon JS, Cook DG. Partial Loss of the Glutamate Transporter GLT-1 Alters Brain Akt and Insulin Signaling in a Mouse Model of Alzheimer's Disease. Pages 509-20. Copyright 2015, with permission from IOS Press.

A large portion of the data and text in Chapter 3 is derived from the following publication:

Meeker KD, Meabon JS, Huber BR, Cook DG. Attenuation of insulin signaling by loss of GLT-1 function in primary cultured neurons is dependent on NMDA receptor activation. *Manuscript in preparation.*

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DEDICATION

To my wife, Jordyn, for all of her love and support.

CHAPTER 1:

Background and Significance

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that manifests in deficits in cognitive function along with alterations in mood and behavior. By advanced stages of the disease, AD patients have severe deficits in cognition and basic functional abilities required for subsistence. Approximately 5.3 million Americans currently have AD and that number is estimated to grow to 11-16 million by 2050 (Alzheimer's-Association, 2015). In 2015, total healthcare costs for dementia patients 65 years of age and older are estimated to be $226 billion (Alzheimer's-Association, 2015). While AD has been listed as the sixth leading cause of death in the United States by the Centers for Disease Control and Prevention (CDC), the number of deaths resulting from the complications of AD is likely much larger (James et al., 2014). Currently, the exact cause(s) of AD is not known but the disease ultimately results in dysfunction and death of neurons in the brain.

Neuropathological characteristics, etiology, and risk factors

Neuropathologically, AD is characterized by deposition of specific protein aggregates, most notably of amyloid β (Aβ) and hyperphosphorylated forms of the microtubule-associated protein tau, which form into amyloid plaques and neurofibrillary tangles, respectively. These changes are accompanied by neuronal death and degeneration of specific brain regions, particularly those important for cognitive function such as the hippocampus and cortex.

Furthermore, synaptic loss is also a prominent component of the disease and is the best correlate of cognitive decline in AD (DeKosky & Scheff, 1990; Selkoe, 2002; Terry et al., 1991).

Early-onset familial AD refers to families with multiple affected individuals and symptoms that manifest typically between 30 and 65 years of age (Bird, 1999 [Updated 2012]). These cases cannot be distinguished clinically or neuropathologically from idiopathic AD, but represent less than 3% of total AD cases (Bird, 1999 [Updated 2012]; Campion et al., 1999). The majority of early-onset familial AD is known to be caused by genetic mutations in any one of three genes (Bird, 1999 [Updated 2012]). These mutations inherited in early-onset familial AD are found in the genes of the amyloid precursor protein (APP, gene: *APP*), presenilin 1 (PS1; gene: *PSEN1*), and presenilin 2 (PS2; gene: *PSEN2*). APP is an integral membrane protein cleaved by the β- (also known as BACE1) and γ-secretases to form the Aβ peptide, which is then found to oligomerize and aggregate into extracellular deposits in AD. PS1 (and its homolog PS2) is a component of the γ-secretase complex. Mutations in *APP* result in increased generation of Aβ (Cai et al., 1993; Citron et al., 1992; Eckman et al., 1997; Haass et al., 1995; Suzuki et al., 1994), while mutations in *PSEN1/PSEN2* result in insufficient digestion of Aβ causing formation of the longer $A\beta_{42}$ peptide, believed to be a more pathogenic form than $A\beta_{40}$ (De Strooper, 2007). Mutations in *APP, PSEN1*, and *PSEN2* are all considered to be fully penetrant, however, there are rare instances of non-penetrance in individuals with *PSEN2* mutations after 80 years of age (Bird, 1999 [Updated 2012]).

In contrast to monogenic forms of AD, the remaining majority of AD cases are idiopathic and the etiology is likely multifactorial. Supporting this, various risk factors for AD have been identified that increase the likelihood for developing the disease. Age is the most prominent risk factor as AD is typically diagnosed in individuals 65 years and older, however, it must be noted

that AD is not a normal component of aging (Alzheimer's-Association, 2015). The next most significant risk factor that has been identified for AD is the apolipoprotein E (*APOE*) gene. The ApoE protein is part of a family of lipoproteins that are responsible for packaging cholesterol and other fats and transporting them through the bloodstream (Saunders et al., 2000; Yu et al., 2014). Three major *APOE* alleles have been identified, ε2, ε3, and ε4, with the ε3 allele being the most common. Inheritance of the ε4 allele has been found to significantly increase the risk of developing AD compared to the ε3 allele, with an even greater risk with inheritance of two ε4 alleles, while having the ε2 allele may even decrease the risk of developing AD (Saunders et al., 2000; Yu et al., 2014). It has been estimated that between 40% and 65% of people diagnosed with AD carry at least one copy of the ε4 allele (Alzheimer's-Association, 2015; Raber et al., 2004; Saunders et al., 1993).

In addition to the risk factors mentioned above, several other diseases and disorders also cause an increased risk of developing AD. Several factors related to cardiovascular disease (obesity, smoking, high cholesterol, and diabetes) increase the risk of developing AD (Ahtiluoto et al., 2010; Anstey et al., 2007; Cheng et al., 2011; Fitzpatrick et al., 2009; Kivipelto et al., 2005; Launer et al., 2000; Ohara et al., 2011; Pendlebury & Rothwell, 2009; Ronnemaa et al., 2011; Rusanen et al., 2010; Solomon et al., 2009; Xu et al., 2011). Moderate and severe traumatic brain injuries (TBI), along with growing evidence for repeated mild TBI, have also been found to significantly increase the risk of developing AD (Plassman et al., 2000) with growing evidence that repeated mild TBI may as well (Gavett et al., 2010; McKee et al., 2013; Monti et al., 2013).

Cellular and molecular changes in Alzheimer's disease

A great deal of research has been conducted to gain an understanding of the cellular and molecular basis of AD yet the exact cause, or potentially causes, of the disease remain unknown. As depositions of Aβ and hyperphosphorylated tau are the primary pathological hallmarks of AD, a lot of attention has been focused on how these two proteins disrupt biological processes occurring in the brain. The focus of this thesis is on glutamatergic signaling alterations, likely resulting from changes in glutamate transporter function, and disturbances in insulin signaling in the brain. Dysfunction in each of these systems has been identified in AD brain tissue with animal models of AD providing supporting evidence of cellular and molecular changes in glutamatergic and insulin signaling. The remainder of this chapter will be used elaborate upon this evidence as well as provide further background information regarding the importance of these systems and how they function under normal conditions in the central nervous system (CNS).

Glutamatergic signaling in the central nervous system

The amino acid glutamate is the primary excitatory neurotransmitter in the CNS and is critical for a variety of functions in the brain including learning and memory. Glutamate also plays essential roles in CNS development, synaptic plasticity, and cell death (Danbolt, 2001; Esposito et al., 2013; Y. Wang & Qin, 2010). Release of glutamate occurs primarily through exocytosis from neuronal axon terminals leading to dramatic increases in extracellular glutamate. Total glutamate concentrations in the brain are estimated to be 5-15 mmol/kg, with the majority of this being found in neurons (Danbolt, 2001; Featherstone, 2010). In glutamatergic neurons, glutamate levels are estimated at approximately 5-10 mM in the cytoplasm (Bramham et al., 1990; Featherstone, 2010; Osen et al., 1995; Ottersen et al., 1990; Ottersen et al., 1992).

However, the ambient extracellular concentration of glutamate must remain very low at baseline, with estimates in the low nanomolar range (Herman & Jahr, 2007), as prolonged exposure to high extracellular glutamate is toxic to neurons and can lead to neuronal dysfunction and death. Furthermore, extracellular glutamate concentration must also be tightly regulated to maintain spatial and temporal resolution of signaling (Bergles et al., 1999).

Glutamate clearance mechanisms

Unlike some other neurotransmitters, such as acetylcholine that relies on extracellular degradation mechanisms to terminate its activity, no extracellular enzymes have been identified that can degrade glutamate. Glutamate is cleared from the extracellular space by transport primarily into astrocytes, although both neurons and oligodendrocytes may contribute to lowering glutamate levels as well. Uptake of extracellular glutamate is carried out predominantly by a family of five high-affinity Na^+-dependent glutamate transporters referred to as GLAST, GLT-1, EAAC1, EAAT4, and EAAT5 (also known as EAAT1 though EAAT5, respectively) (Danbolt, 2001; Y. Zhou & Danbolt, 2014).

All of the glutamate transporters couple the transport of substrate to the exchange of $1H^+$ and $3Na^+$ for $1K^+$ (Klockner et al., 1993; Levy et al., 1998; Owe et al., 2006; Zerangue & Kavanaugh, 1996). Sodium is required for the binding of glutamate while potassium is required for net transport. Without potassium, net uptake cannot occur, but exchange of external and internal substrate can still occur at a 1:1 ratio (Danbolt & Storm-Mathisen, 1986; Kanner & Bendahan, 1982; Kanner & Sharon, 1978; Otis & Kavanaugh, 2000; Pines & Kanner, 1990). Although heteroexchange does not have an effect on glutamate clearance as net uptake is unchanged, it is an important property to consider when applying inhibitors. For example, glutamate transporter inhibitors that are transportable may actually lead to release of glutamate

due to heteroexchange with the inhibitor. Transport of L-glutamate, D-aspartate, and L-aspartate occur with similar affinities while the transporters have a very low affinity for D-glutamate (Danbolt, 2001; Y. Zhou & Danbolt, 2014).

Not only do glutamate transporters regulate glutamate action through removal from the extracellular space by uptake, but they also bind and buffer glutamate, particularly EAAC1 (Scimemi et al., 2009; Tzingounis & Wadiche, 2007). Glutamate buffering prolongs the time course of clearance by uptake and prevents its ability to diffuse outside of the synapse, which may reduce the recruitment of extrasynaptic glutamate receptors (Scimemi et al., 2009; Tzingounis & Wadiche, 2007). Glutamate transporters can also function as chloride channels, which is thermodynamically uncoupled from transport (Fairman et al., 1995; Gameiro et al., 2011; Mim et al., 2005; Ryan & Mindell, 2007; Wadiche et al., 1995). EAAT4 and EAAT5 in particular have been hypothesized to function more as inhibitory glutamate receptors than as transporters (Dehnes et al., 1998; Schneider et al., 2014; Veruki et al., 2006).

Glutamate transporters are integral membrane proteins distributed differentially throughout the brain. GLAST is selectively localized to astrocytes in the CNS (Lehre et al., 1995), shows high expression in the cerebellum, the retina (Lehre et al., 1997; Rauen et al., 1996; Rauen et al., 1998), the inner ear (Furness & Lehre, 1997; Takumi et al., 1997), and the circumventricular organs (Berger & Hediger, 2000). GLT-1 expression is predominantly localized to astrocytes, but about 10% or less of GLT-1 is found in neuronal axon terminals (Furness et al., 2008). Intriguingly, despite this substantial difference in expression levels, uptake by GLT-1 was found to be as fast in axon terminals as in astrocytes (Furness et al., 2008). GLT-1 expression is most prominent in forebrain regions, where it represents approximately 1% of total protein. For example, in the hippocampus, GLT-1 levels are roughly four times higher than

GLAST (Lehre & Danbolt, 1998). EAAC1 expression in the CNS is highest in the hippocampus, although at levels approximately 100 times lower than GLT-1, where it is selectively expressed in neurons (Holmseth et al., 2012). Subcellularly, EAAC1 is localized to dendrites and the cell soma but not axon terminals (Holmseth et al., 2012; Shashidharan et al., 1997). Thus, in neurons, GLT-1 and EAAC1 are localized to distinct sites. Furthermore, GLAST, GLT-1, and EAAC1 are each believed to form homotrimers by non-covalent linking of subunits (Haugeto et al., 1996). EAAT4 is predominantly expressed in dendritic spines of cerebellar Purkinje cells (Dehnes et al., 1998; Fairman et al., 1995), with some EAAT4 also found in a subset of forebrain neurons (de Vivo et al., 2010; Dehnes et al., 1998; Massie et al., 2008). EAAT5 is almost exclusively expressed in the retina with brain levels of EAAT5 being very low (Arriza et al., 1997; Eliasof et al., 1998).

GLT-1 function is critical for survival, which was identified in GLT-1 knockout mice that showed hippocampal CA1 neuron loss and severe, spontaneous seizures resulting in 50% mortality by 6 weeks of age (Tanaka et al., 1997). These findings are consistent with the high forebrain expression levels of GLT-1 as compared to the other transporters. Moreover, others have shown that GLT-1 is responsible for greater than 90% of total glutamate uptake in the brain (Danbolt et al., 1992; Haugeto et al., 1996). The phenotypes associated with GLAST, EAAC1, or EAAT4 deficiency, however, are far less severe. Mice lacking GLAST develop normally but show motor discoordination, hearing loss after acoustic overstimulation, and increased susceptibility to cerebellar and retinal damage in injury models consistent with the predominant expression of GLAST in the affected regions (Hakuba et al., 2000; Harada et al., 1998; Watase et al., 1998). There are also unpublished reports that GLAST knockout mice are obese (personal communication, K. Tanaka). While GLAST deficiency on its own does not result in spontaneous

seizures, it does increase seizure duration and intensity (T. Watanabe et al., 1999). Moreover, combined GLT-1 and GLAST loss results in defects in brain development and perinatal mortality (Matsugami et al., 2006). Glutamate transporters, particularly EAAC1, are also the primary method for cysteine uptake into mature neurons, which is the precursor of the antioxidant glutathione (Aoyama et al., 2006). Loss of EAAC1 *in vivo* leads to age-dependent neurodegeneration likely due to a depletion in neuronal glutathione content (Aoyama et al., 2006). EAAT4 deficient mice appear normal although they show some alterations in cerebellar signaling (Huang et al., 2004) and EAAT5 knockout mice appear to have been developed although very little information on them currently exists (see Mouse Genome Informatics database entry for gene *SLC1A7*).

Glutamate transporter dysfunction and loss in Alzheimer's and other diseases

Supporting the critical role of glutamate throughout the CNS, it is not surprising that dysfunction of glutamate transporters, the primary regulators of glutamate action, are implicated in several neurological diseases and disorders. For example, human GLAST mutations have been identified in episodic ataxia cases (de Vries et al., 2009; Jen et al., 2005) and schizophrenia patients (T. Walsh et al., 2008). Several studies have found EAAC1 polymorphisms to be associated with obsessive-compulsive disorders (Arnold et al., 2006; Dickel et al., 2006; Shugart et al., 2009; Stewart et al., 2007). In addition, EAAC1 deficiency in humans also results in dicarboxylic aminoaciduria due to the role of EAAC1 in kidney function (Bailey et al., 2011). Finally, loss of cerebellar EAAT4 has been identified in humans with spinocerebellar ataxia type 5 (SCA5; also known as Lincoln's ataxia) (Ikeda et al., 2006).

In keeping with its dominant role in forebrain glutamate uptake, GLT-1 has been implicated in the widest variety of CNS disorders and insults, including AD, and thus is the focus

of this thesis. Although no GLT-1 (typically referred to as EAAT2 or its corresponding gene name of *SLC1A2* in human studies) mutations in humans have been identified, several disorders are accompanied by dysfunction or loss of GLT-1. As mentioned earlier, loss of GLT-1 *in vivo* results in spontaneous seizures (Tanaka et al., 1997) and overexpression of GLT-1 in a model of status epilepticus reduced chronic mortality rate, chronic seizure frequency, and hippocampal neuron degeneration (Kong et al., 2012), implicating GLT-1 in epilepsy and as a potential therapeutic target. Furthermore, increased extracellular glutamate and alterations in the mRNA and protein expression of GLT-1 have been identified in the hippocampi of temporal lobe epilepsy patients (During & Spencer, 1993; Proper et al., 2002). Similarly, excessive glutamate release has been identified in ischemic stroke and both genetically and pharmacologically-induced (using the β-lactam antibiotic ceftriaxone) increased expression of GLT-1 is neuroprotective (Chu et al., 2007; Harvey et al., 2011; Rothstein et al., 2005). However, others have identified that glutamate transport can be reversed during ischemia leading to glutamate release through transporters (Rossi et al., 2000). In both sporadic and familial amyotrophic lateral sclerosis (ALS) patients there is a loss of GLT-1 in the motor cortex and spinal cord (Bristol & Rothstein, 1996; Rothstein et al., 1995). Animal models of ALS expressing SOD1 familial mutations also show reductions in GLT-1 expression (Bendotti et al., 2001; Bruijn et al., 1997). GLT-1 mRNA has been found to be reduced in Huntington's disease (HD) brains and was negatively correlated with disease severity (Arzberger et al., 1997; Faideau et al., 2010). Increasing the expression of GLT-1 with ceftriaxone improves the behavioral phenotype induced in mouse models of HD (Miller et al., 2012; Miller et al., 2008). Moreover, GLT-1 levels have been found to be reduced in the cerebellar cortex of individuals with essential tremor (M. Lee et

al., 2014). TBI also reduces GLT-1 levels in both humans and animal models, which can be restored by ceftriaxone treatment (Goodrich et al., 2013; van Landeghem et al., 2006).

As discussed earlier, there is evidence that glutamatergic dysfunction plays a role in AD pathogenesis. Consistent with this, several studies including work from our lab have implicated GLT-1 loss or dysfunction in AD. There is evidence that GLT-1 becomes oxidatively damaged (Lauderback et al., 2001) and several studies have found GLT-1 levels to be significantly reduced, by as much as 50%, in AD (Scott et al., 2011; Woltjer et al., 2010). Similar to aggregation of Aβ and tau in AD, GLT-1 has also been found to aberrantly accumulate into detergent-insoluble complexes in the hippocampus and cerebral cortex, which correlated with the level of cognitive impairment (Woltjer et al., 2010). There is also a growing body of evidence suggesting that epileptic seizures are a component of AD, which is consistent with the loss of GLT-1 seen in the disease. It has been estimated that 10 to 22% of AD patients experience at least one episode of an unprovoked seizure (Mendez & Lim, 2003). Other studies have also shown an increased occurrence of seizures in AD patients, including early-onset familial cases, compared to the general population (Amatniek et al., 2006; Hesdorffer et al., 1996).

In *ex vivo* models of AD, synaptic changes induced by Aβ treatment were found to be mimicked by inhibition of glutamate transport (S. Li et al., 2009; S. Li et al., 2011). These findings are consistent with the hypothesis that Aβ impairs GLT-1 function, which leads to synaptic and neuronal impairment. Furthermore, studies have also identified a role of GLT-1 in brain metabolism by regulating activity-dependent glucose utilization (Voutsinos-Porche et al., 2003) and GLT-1 can be modulated by components of the insulin signaling cascade (Ji et al., 2011; L. B. Li et al., 2006; Wu et al., 2010), a pathway known to be disturbed in AD. This may

suggest an interaction between GLT-1 dysfunction and insulin signaling disturbances in AD, which will discussed further later in this chapter.

Glutamate receptor localization and signaling characteristics

Along with the critical regulatory role that glutamate transporters play in glutamate action, glutamate receptors are the other key component determining the signaling properties of released glutamate. Two families of glutamate receptors have been identified in the CNS, ionotropic glutamate receptors and metabotropic glutamate receptors. Ionotropic glutamate receptors conduct ions through a channel pore upon activation whereas metabotropic glutamate receptors are G protein-coupled receptors (GPCR) that use intracellular G proteins to transmit signals upon activation by glutamate. There are three subfamilies of ionotropic glutamate receptors that exist including: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and kainate receptors. Metabotropic glutamate receptors (mGluR) are also categorized into three subfamilies denoted as group I, II, or III, which together comprise a total of 12 mGluRs encoded by eight genes (Willard & Koochekpour, 2013). Most, if not all, cells in the CNS express at least one type of glutamate receptor (Y. Zhou & Danbolt, 2014).

NMDA receptors in particular will be the focus of this thesis due to their implication in AD pathogenesis. As with the other ionotropic glutamate receptors, NMDA receptors are heterotetrameric ligand-gated ion channels but have distinct properties including: an ion channel that is blocked by Mg^{2+} in a voltage-dependent manner; they are highly permeable to Ca^{2+}; they display slow kinetics due to slow glutamate unbinding; and finally, activation of the receptor requires glutamate and a co-agonist of glycine or D-serine (Paoletti et al., 2013; Traynelis et al., 2010). The four subunits that comprise the receptor share a similar structure consisting of four

domains: the extracellular amino-terminal, the extracellular ligand-binding domain, the transmembrane domain, and the intracellular carboxy-terminal (Paoletti et al., 2013; Traynelis et al., 2010; Willard & Koochekpour, 2013). Functional NMDA receptors require assembly of two GluN1 (also known as NR1) subunits with two GluN2 (also known as NR2) subunits (Furukawa et al., 2005; Laube et al., 1998; Monyer et al., 1992; Schorge & Colquhoun, 2003). NMDA receptors can also be formed with two GluN1 subunits and a combination of GluN2 and GluN3 (also known as NR3) subunits, but GluN3 subunits do not form functional receptors on their own (Chatterton et al., 2002; Ulbrich & Isacoff, 2007, 2008; Yao & Mayer, 2006). The GluN1 and GluN3 subunits provide the glycine binding sites and the GluN2 subunits provide glutamate binding sites (Furukawa & Gouaux, 2003; Furukawa et al., 2005; Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Lerma et al., 1990; Yao et al., 2008).

Seven different NMDA receptor subunits have thus far been identified: the GluN1 subunit, four GluN2 subunits denoted as GluN2A-D, and two GluN3 subunits denoted as GluN3A and B, each encoded by its own gene (Paoletti et al., 2013). GluN1 subunits are ubiquitously expressed throughout the CNS from early development to adulthood (Monyer et al., 1994; M. Watanabe et al., 1992). The GluN2 subunits, however, are the major determinants of the NMDA receptors functional properties and localization. In the adult brain, GluN2A (NR2A) expression is widely expressed throughout the majority of the CNS whereas GluN2B (NR2B) expression is restricted to forebrain regions (Monyer et al., 1994; M. Watanabe et al., 1992). GluN2C (NR2C) is expressed mainly in the cerebellum and olfactory bulb while GluN2D (NR2D) is expressed at low levels in the adult brain, predominantly in the diencephalon and mesencephalon (Monyer et al., 1994; M. Watanabe et al., 1992). While the di-heteromeric GluN1/GluN2B and GluN1/GluN2A receptor compositions found in the adult CNS represent a

significant fraction of NMDA receptors (Al-Hallaq et al., 2007), it is also possible for the two non-GluN1 subunits to be different from each other, such as with two different GluN2 subunits or a GluN2 subunit with a GluN3 subunit. Tri-heteromeric GluN1/GluN2A/GluN2B receptors have been identified in several brain regions, particularly the hippocampus and cortex, and may represent a significant portion of the receptor population (Rauner & Kohr, 2011). Other tri-heteromeric receptor assemblies have been identified as well, but in contrast to di-heteromeric receptors, much less is known about the functional properties of tri-heteromeric NMDA receptors.

Along with expression patterns, NMDA receptor subtypes can also provide different subcellular localization. GluN2A-containing receptors are typically localized to synaptic sites while GluN2B-containing receptors are prominently located at peri- and extrasynaptic sites (Groc et al., 2006; Martel et al., 2009; Parsons & Raymond, 2014; Tovar & Westbrook, 1999). However, it must be noted that NMDA receptors containing either GluN2A or GluN2B have not been found to be exclusively confined to these sites (A. Z. Harris & Pettit, 2007; X. B. Liu et al., 2004; Petralia et al., 2010; Thomas et al., 2006). Subunit composition also determines the functional properties of the NMDA receptor, including single-channel conductance, Mg^{2+} blockade, Ca^{2+} permeability, maximal channel open probability, agonist sensitivity, and deactivation kinetics (Paoletti et al., 2013; Traynelis et al., 2010). For example, GluN2A- and GluN2B-containing receptors both show high conductance, high sensitivity to Mg^{2+}, and high Ca^{2+} permeability while GluN2C- and GluN2D-containing receptors have lower conductances, sensitivity to Mg^{2+}, and Ca^{2+} permeability (Paoletti et al., 2013; Traynelis et al., 2010). Each type of subunit also confers very different properties for glutamate binding and receptor deactivation kinetics with affinity for glutamate being GluN2D > GluN2C > GluN2B > GluN2A (with a

similar pattern for glycine) and the glutamate deactivation time constant being GluN2D > GluN2C = GluN2B > GluN2A (Paoletti et al., 2013; Vicini et al., 1998). Thus, GluN2A-containing receptors have a lower affinity for glutamate and shut off much more rapidly than GluN2D-containing receptors. Furthermore, GluN2A-containing receptors have a higher open probability than GluN2B- containing receptors and a much higher open probability than GluN2C- and GluN2D-containing receptors (Paoletti et al., 2013).

Glutamate receptor expression and signaling abnormalities in Alzheimer's disease

As discussed, glutamate transporter dysfunction/loss appears to be a consistent and prominent component of AD. Along with this, other components of the glutamatergic system have also been found to be perturbed in human AD cases and animal models of AD. Reduced levels of the vesicular glutamate transporters (VGLUT) 1 and 2, which are responsible for returning glutamate back to synaptic vesicles, have been identified in the prefrontal cortex of individuals with AD (Kashani et al., 2008; Kirvell et al., 2006). It has also been shown that Aβ predominantly accumulates in terminals expressing VGLUT1/2 over others (Sokolow et al., 2012), suggesting that it may preferentially target presynaptic glutamatergic terminals. Synaptic loss has been identified as the strongest pathological correlate of cognitive decline in AD (DeKosky & Scheff, 1990; Terry et al., 1991) with evidence that glutamatergic terminals are the most susceptible to Aβ-induced loss (Canas et al., 2014). Evidence for changes in ionotropic glutamate receptor expression and function in AD patients is not as straight-forward, but most reports have identified decreases in the protein and mRNA levels of these receptors, particularly NMDA receptors (Hynd et al., 2004a, 2004b; Panegyres et al., 2002; Sze et al., 2001). Furthermore, alterations in mGluR and AMPA receptor binding and expression have been identified in AD (Albasanz et al., 2005; Dewar et al., 1991; H. G. Lee et al., 2004).

While glutamatergic dysfunction in human AD cases has yet to be conclusively demonstrated, work in AD models has provided significant evidence linking Aβ and tau to glutamatergic dyshomeostasis (Crimins et al., 2013; Esposito et al., 2013; Revett et al., 2013). Glutamate receptors are believed to conduct their role in learning and memory through processes known as long-term potentiation (LTP) and long-term depression (LTD) through which synapses are either strengthened and increased or weakened and removed, respectively. Aβ oligomers, including those extracted from AD brains, decrease LTP, increase LTD, and reduce dendritic spine density when applied to rodent hippocampal slices (S. Li et al., 2009; S. Li et al., 2011; Shankar et al., 2007; Shankar et al., 2008; H. W. Wang et al., 2002). Transgenic animal models that express human APP with familial AD mutations exhibit dendritic spine loss near plaque deposits (Lanz et al., 2003; Moolman et al., 2004; Spires et al., 2005; Tsai et al., 2004). Furthermore, transgenic mutant APP and PS1 mice develop synaptic dysfunction before significant plaque formation (Chapman et al., 1999; D'Amelio et al., 2011; Fitzjohn et al., 2001; Games et al., 1995; Trinchese et al., 2004) and injection of oligomeric Aβ into the brains of rodents causes spine loss, impaired LTP, and behavioral impairments (Cleary et al., 2005; Cullen et al., 1997; Kim et al., 2001; Shankar et al., 2008; D. M. Walsh et al., 2002). Together, these findings suggest that soluble, oligomeric forms of Aβ, rather than plaques, are responsible for synaptic dysfunction.

NMDA receptor activation was found to be required for spine loss and other synaptic changes induced by Aβ (Roselli et al., 2005; Shankar et al., 2007; Shankar et al., 2008). GluN2B-containing NMDA receptors in particular have been identified as being responsible for impairment of synaptic plasticity (Bordji et al., 2010; Kervern et al., 2012; S. Li et al., 2011), suggesting a role of extrasynaptic NMDA receptors in Aβ-induced synaptic dysfunction. In

keeping with these findings, NMDA receptor antagonists, including GluN2B-specific antagonists, were found to prevent spine loss (S. Li et al., 2011; Wei et al., 2010). Interestingly, many of the effects of Aβ on NMDA receptor activation and resulting synaptic impairment were found to be mimicked by inhibition of glutamate transport (S. Li et al., 2009; S. Li et al., 2011). Aberrant levels of Aβ have also been shown to negatively regulate general synaptic transmission by inducing endocytosis of NMDA and AMPA receptors from the postsynaptic membranes (Hsieh et al., 2006; Kurup et al., 2010; Snyder et al., 2005; W. Q. Zhao et al., 2010).

In conjunction with synaptic dysfunction, Aβ is also believed to induce glutamate-mediated excitotoxicity, which occurs through overstimulation of NMDA receptors resulting in activation of cell death signaling. How Aβ leads to glutamate-mediated excitotoxicity has not been fully resolved but there is evidence that it can bind directly to NMDA and AMPA receptors, leading to elevated intracellular Ca^{2+} (Alberdi et al., 2010). NMDA and AMPA receptor antagonists can prevent Aβ-induced cell death. Furthermore, as discussed, Aβ can cause dysfunctional glutamate transport and/or increased glutamate release resulting in elevated extracellular glutamate levels and excitotoxicity (Fernandez-Tome et al., 2004; M. E. Harris et al., 1996; Kabogo et al., 2010; Noda et al., 1999).

In contrast to Aβ, the cellular mechanisms by which tau contributes to synaptic and neuronal degeneration are less clear, but there is evidence that tau may also cause synaptic dysfunction and loss through glutamatergic signaling alterations. Similar to Aβ, recent data has shown that soluble forms of tau, as opposed to tangles, are the toxic species (Kopeikina et al., 2012; Rocher et al., 2010). Mislocalization of phosphorylated tau to dendritic spines in neurons cultured from rTg4510 mice, which express a mutant form of human tau, is associated with decreased AMPA and NMDA receptor synaptic expression (Hoover et al., 2010). However,

recent studies examining the consequences of tau pathology on excitatory synaptic responses have provided conflicting results regarding baseline glutamatergic transmission and LTP induction in hippocampal neurons of various models of tauopathy (Boekhoorn et al., 2006; Hoover et al., 2010; Schindowski et al., 2006; Sydow et al., 2011; Yoshiyama et al., 2007).

Given its role as a microtubule stabilizing protein, tau is likely to contribute to synaptic dysfunction and neuronal death through impairment of axonal transport and neuritic dystrophy, which is supported by findings in human tauopathies and AD (Braak & Braak, 1997) as well as in animal models (Ishihara et al., 1999; Leroy et al., 2007; W. L. Lin et al., 2003; Ludvigson et al., 2011; Probst et al., 2000; Spittaels et al., 1999). Consistent with this, abnormally phosphorylated tau impairs trafficking or anchoring of glutamate receptors to synapses, which contributes to synaptic dysfunction (Hoover et al., 2010). However, tau likely also contributes to Aβ toxicity as inhibition of tau phosphorylation prevents Aβ-induced neurodegeneration and neurons cultured from tau knockout mice are resistant to Aβ toxicity (Alvarez et al., 2002; Rapoport et al., 2002; Zheng et al., 2002).

While evidence of glutamatergic signaling dysfunction in humans is currently limited, there is a wealth of evidence in animal models of AD that Aβ, and likely tau, disturb synaptic function through alterations in the glutamatergic system (Crimins et al., 2013; Danysz & Parsons, 2012; Esposito et al., 2013; Revett et al., 2013). Interestingly, the low-affinity, uncompetitive NMDA receptor antagonist memantine is one of the few drugs approved for the treatment of AD suggesting that the glutamatergic system may be a good target for therapeutic intervention. Given the evidence of reduced GLT-1 expression and function at early stages of AD, it may also prove to be beneficial to more directly target extracellular glutamate by modulating glutamate uptake. Several drugs have been identified that induce expression of GLT-

1 and prevent reductions in GLT-1 levels in disease models such as ceftriaxone (Miller et al., 2008; Rothstein et al., 2005), minocycline (Nie et al., 2010), tamoxifen (Karki et al., 2013), and LDN/OSU-0212320 (Kong et al., 2014).

Insulin signaling in the central nervous system

In addition to dysfunction in glutamatergic uptake and signaling in AD, there is a growing body of evidence suggesting that insulin signaling disturbances may play a role in AD. Insulin is a peptide released from the pancreas that regulates metabolism of glucose, fats, and proteins. Most of the research on insulin has focused on its roles in the periphery including the liver, muscle, and adipose tissue. However, in recent years, insulin signaling in the CNS has become more appreciated. In the brain, insulin regulates a diverse set of functions including food intake, body weight, mood, neuronal survival, dendritic outgrowth, synaptic plasticity, memory, and cognitive function (Bassil et al., 2014; Fernandez & Torres-Aleman, 2012; Kleinridders et al., 2014; McNay & Recknagel, 2011).

While the exact mechanism of how insulin gets into the brain still remains controversial, insulin circulating in the blood can cross the blood-brain barrier (BBB) through a receptor-mediated active transport system (Frank et al., 1985; Pardridge et al., 1985). This pathway is consistent with studies showing that insulin levels in the cerebrospinal fluid (CSF) increase proportionally with blood insulin after peripheral insulin infusion (Banks et al., 1997; Baura et al., 1993; Margolis & Altszuler, 1967). There is also evidence that insulin can be directly synthesized and secreted by neurons (Clarke et al., 1986; Devaskar et al., 1994) and insulin mRNA has been found in rat, rabbit, and human brains (Devaskar et al., 1994; Devaskar et al., 1993; Rivera et al., 2005; Steen et al., 2005). However, the amount of insulin produced in the brain and whether this pool of insulin is physiologically relevant still remains elusive. It is

possible that both the centrally and peripherally derived pools of insulin are important for signaling in the brain.

Insulin receptor expression is distributed throughout the brain with the highest expression levels identified in the olfactory bulb, cortex, hippocampus, hypothalamus, and cerebellum with relatively low levels in the striatum, thalamus, midbrain, and brainstem (Fernandez & Torres-Aleman, 2012; Havrankova et al., 1978; Unger et al., 1989). In the brain, insulin receptors are present at low levels in glial cells and much higher concentrations in neurons, where they preferentially localize to the postsynaptic density (Abbott et al., 1999; Unger et al., 1989). The insulin receptor consists of two extracellular α-subunits and two transmembrane β-subunits, which are encoded by the same gene and each have a lower molecular weight than peripheral insulin receptor subunits likely due to differential transcriptional or translational regulation (Goldstein & Dudley, 1992; Heidenreich et al., 1983; Sugimoto et al., 2000).

Insulin binds to the α-subunit resulting in autophosphorylation of tyrosine residues in the β-subunit kinase domain (Figure 1.1). This allows for recruitment of proteins with Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, most prominently the insulin receptor substrate 1 (IRS-1) protein. Activation of IRS-1 results in signaling to phosphoinositide 3-kinase (PI3K) and downstream activation of the serine/threonine kinase Akt (also known as protein kinase B; PKB). Akt regulates numerous proteins involved in a variety of cellular processes, most notably glycogen synthase kinase-3β (GSK-3β) and mammalian target of rapamycin (mTOR), which in the brain regulate protein synthesis/autophagy and neuronal progenitor cell proliferation/neuronal polarity/neuroplasticity, respectively. Insulin signaling can also activate the Ras/extracellular signal-regulated kinase (ERK) pathway (Bassil et al., 2014; Fernandez & Torres-Aleman, 2012; Kleinridders et al., 2014; McNay & Recknagel, 2011).

Metabolic functions of insulin signaling in the brain

The most recognized role of peripheral insulin is to promote cellular uptake of glucose by promoting the recruitment of vesicles containing the glucose transporters to the cell membrane. This role is well characterized in the periphery, such as skeletal muscle and adipose tissue, while the brain was previously believed to be an insulin-insensitive tissue. However, more recent evidence suggests that insulin may modulate neuronal glucose uptake, although likely to a limited extent. GLUT4, the insulin-sensitive glucose transporter, mRNA is expressed in brain regions that show the highest expression of the insulin receptor (El Messari et al., 2002; Vannucci et al., 1998) and insulin treatment has been shown to induce translocation of GLUT4 to the cell surface of hippocampal neurons (Grillo et al., 2009; Piroli et al., 2007). Insulin has also been found to promote translocation of another glucose transporter, GLUT3, to the cell surface of hippocampal neurons, which resulted in increased glucose uptake, but only under conditions of increased neuronal activity (Uemura & Greenlee, 2006).

In vivo, several studies have shown that insulin can regulate glucose uptake and metabolism in the brain (Bingham et al., 2002; Henneberg & Hoyer, 1994; Hoyer et al., 1996), which are attenuated in a T2DM animal models (McNay et al., 2010; Winocur et al., 2005). Conversely, it has been shown using *ex vivo* hippocampal slices and synaptosomes from human normal control and AD patients that insulin stimulation on its own does not influence glucose uptake while stimulation with glutamate significantly increased glucose uptake (Talbot et al., 2012). However, it was not determined if glutamate-induced glucose uptake could be further modulated by insulin stimulation. Thus, while neurons can acquire glucose in an insulin-independent manner, insulin may be able to induce even greater uptake of glucose during periods of high neuronal activity to maintain a sufficient energy supply. Together these findings may

suggest a cooperative role between glutamate and insulin action in regulating neuronal metabolism.

In addition to direct effects on brain metabolism, several studies have shown that insulin action in the CNS can lead to regulation of peripheral metabolism. It was initially identified that intracerebroventricular (ICV) administration of insulin in baboons caused dramatic decreases in food intake and body weight (Woods et al., 1979). Infusion of insulin in the third ventricle of rodents results in decreased food intake through reductions in the levels of orexigenic (neuropeptide Y, NPY; Agouti-related peptide, AgRP) and elevations in the levels of anorexigenic (proopiomelanocortin, POMC; cocaine- and amphetamine –regulated transcript, CART) neuropeptides in the hypothalamus (Kleinridders et al., 2014). To further examine the specific role of insulin in the CNS, neuron-specific insulin receptor knockout (NIRKO) mice were developed. In this study they found that both male and female mice developed diet-sensitive obesity, increased plasma leptin levels, mild insulin resistance, elevated plasma insulin levels, and hypertriglyceridemia (Bruning et al., 2000). Furthermore, female NIRKO mice also showed increased food intake. Collectively, these findings support a role for CNS insulin signaling in regulation of peripheral metabolism, likely through insulin action in the hypothalamus.

Insulin signaling in synaptic plasticity and cognitive function

In conjunction with its role in metabolic regulation, there is also evidence that insulin signaling in the CNS is important for neuronal synaptic function, memory, and cognition. Some of the earliest evidence for a role of CNS insulin signaling in cognitive processes came from a study showing that training on a spatial memory task increased hippocampal insulin receptor expression (W. Zhao et al., 1999). In keeping with this, several studies have shown that

administration of exogenous insulin through several routes enhances cognitive function and spatial memory in animals and humans, including by ICV (Haj-ali et al., 2009; Park et al., 2000), intranasally (Benedict et al., 2004; Reger et al., 2006; Reger, Watson, Green, Baker, et al., 2008; Reger, Watson, Green, Wilkinson, et al., 2008), and intrahippocampally (Babri et al., 2007; McNay et al., 2010; Moosavi, Naghdi, & Choopani, 2007; Moosavi et al., 2006; Moosavi, Naghdi, Maghsoudi, et al., 2007). Moreover, both animal models and humans with diabetes show deficits in cognitive function (Biessels et al., 1996; Bruehl et al., 2009; McNay & Recknagel, 2011; Tan et al., 2011).

Genetic deletion of neuronal insulin receptors in the NIRKO mice resulted in significant reductions in Akt and GSK-3β phosphorylation, and an increase in tau phosphorylation, consistent with studies showing that GSK-3β can directly phosphorylate tau (Schubert et al., 2004). However, NIRKO mice do not show alterations in neuronal proliferation/survival or memory, as might be expected given other evidence of insulin signaling as a regulator of cognitive function and with tau hyperphosphorylation being implicated in AD pathogenesis (Schubert et al., 2004). Although these results could suggest roles for insulin like growth factor-1 (IGF-1) or IGF-2 rather than insulin in regulating cognitive function, IGF-1 does not mimic the effects of insulin when given intrahippocampally (McNay et al., 2010). Furthermore, it has also been shown that specific blockade of insulin using an antibody-like peptide that does not have cross-reactivity for IGF-1 or IGF-2 significantly impaired memory (McNay et al., 2010). Thus, there is strong evidence that insulin specifically plays a role in regulating memory and cognitive function.

At the cellular and subcellular levels, insulin has also been found to regulate synaptic plasticity. Insulin can modulate LTP and LTD, cellular correlates of learning and memory,

through modulation of glutamatergic signaling. For example, insulin can induce LTD through AMPA receptor internalization and regulates LTP through an NMDA receptor-dependent mechanism, possibly by promoting delivery of NMDA receptors to the cell surface (Ahmadian et al., 2004; J. W. Lin et al., 2000; Man et al., 2000; Skeberdis et al., 2001; van der Heide et al., 2005).

Evidence of central nervous system insulin resistance in Alzheimer's disease

In conjunction with the other pathological hallmarks of AD that have been discussed, there has been increasing evidence of disturbed insulin signaling in the brains and periphery of AD patients. Epidemiological studies show that having T2DM can lead to cognitive dysfunction and increased risk of developing AD, suggesting that peripheral and central metabolic changes are closely related (Arvanitakis et al., 2004; Brands et al., 2007; Leibson et al., 1997; Ott et al., 1999; Schrijvers et al., 2010; Xu et al., 2004). In keeping with this, AD patients have reduced CSF insulin levels while plasma insulin levels are elevated (Craft et al., 1998). AD patients also show impaired insulin-mediated glucose disposal, a pattern consistent with peripheral insulin resistance (Craft et al., 1999). Moreover, T2DM and AD mouse models show similar changes including deficits in cognitive function, vascular dysfunction, mitochondrial impairment, and increased Aβ burden in the cortex and hippocampus (Carvalho et al., 2012; Carvalho et al., 2013). Finally, AD transgenic mice that received a high fat diet show peripheral insulin resistance in conjunction with reduced insulin receptor signaling in the brain, elevated hippocampal amyloid burden, and increased cognitive deficits compared to AD mice maintained on a normal diet (Ho et al., 2004).

Several insulin signaling abnormalities have been identified in AD cases that are consistent with a form of brain insulin resistance. For example, several studies have found

reduced phosphorylated and/or total insulin receptor levels in AD cases compared to normal controls (Rivera et al., 2005; Steen et al., 2005; Talbot et al., 2012). Insulin receptor expression in AD cortical neurons has also been found to localize intracellularly with reduced dendritic staining (Moloney et al., 2010). Furthermore, there is substantial evidence that IRS-1 is more likely to be in an inactivated state in AD brains, identified by increased phosphorylation at several different serine sites including Ser^{312}, Ser^{616}, and $Ser^{636/639}$ (equivalent to Ser^{307}, Ser^{612}, and $Ser^{632/635}$ in rodents) (Bomfim et al., 2012; Moloney et al., 2010; Talbot et al., 2012). Reduced total IRS-1 levels have also been identified in AD patients (Moloney et al., 2010), which is another mechanism used to shut down insulin signaling and a marker of insulin resistance in other models (Boura-Halfon & Zick, 2009).

Downstream in the pathway, several alterations in other insulin signaling-related proteins have been identified in AD. Akt phosphorylation and activity are significantly elevated in the brains of AD patients (Griffin et al., 2005; Pei et al., 2003; Rickle et al., 2004; Talbot et al., 2012). Consistent with this, one of the downstream targets of Akt, mTOR, also shows increased phosphorylation in AD brains (Griffin et al., 2005; X. Li et al., 2005; Talbot et al., 2012). While much attention has been paid to the GSK-3β hypothesis of AD as GSK-3β is one of the kinases can that can directly phosphorylate tau, studies have found mixed results regarding the levels of GSK-3β phosphorylation and activity in AD cases (Griffin et al., 2005; Pei et al., 1997; Steen et al., 2005; Talbot et al., 2012). Consistent with the role of CNS insulin signaling in cognitive function, basal activation states of several insulin signaling proteins were found to be related to episodic memory. Phosphorylation of sites that lead to attenuation of insulin signaling were negatively correlated with memory while phosphorylation of sites that promote insulin signaling were positively correlated with memory (Talbot et al., 2012).

While increases in the activation profiles of downstream insulin signaling proteins (i.e. Akt and mTOR) may seem contradictory to the reductions in the upstream activity of insulin signaling proteins (insulin receptor and IRS-1), it has been suggested that other pathways may influence downstream insulin signaling proteins to induce negative feedback regulation of upstream insulin signaling proteins (Boura-Halfon & Zick, 2009). For example, inflammatory signaling by tumor necrosis factor α (TNFα) can lead to increased activation of Akt or its downstream proteins, such as mTOR or p70 S6 kinase (p70S6K), which results in negative-feedback regulation of insulin signaling through inhibition of the insulin receptor and IRS-1 (Morisco et al., 2005; Ozes et al., 2001; Tian, 2005; Zhang et al., 2008). Aβ is known to induce inflammatory signaling, including through TNFα/JNK, which has been shown to result in increased IRS-1 serine phosphorylation (Bomfim et al., 2012). Furthermore, increased activation of serine kinases known to target IRS-1 (including Akt, GSK-3β, mTOR, and JNK) are found to be increased in CA1 neurons of AD cases and correlate significantly with the elevated levels of IRS-1 serine phosphorylation (Talbot et al., 2012).

While the majority of studies examining insulin signaling in AD tissue have looked at basal phosphorylation states of these proteins, one study (Talbot et al., 2012) examined both basal phosphorylation states of insulin signaling proteins as well as the ability of insulin to signal through the pathway by using hippocampal slices from AD patients. While they found similar basal insulin signaling changes as others previously, insulin-evoked phosphorylation was reduced throughout the insulin signaling pathway including the insulin receptor, IRS-1 (tyrosine phosphorylation), Akt, GSK-3β, and mTOR (Talbot et al., 2012). Collectively, these results suggest the ability of insulin to stimulate its signaling pathway is reduced in the CNS in AD, considered a state of insulin resistance in the brain. While basal activation of downstream

elements of the insulin signaling cascade appears to be elevated in AD brains, this may be due to signals from other pathways actually promoting further brain insulin resistance through negative feedback regulation as downstream insulin signaling proteins actually show blunted responses to insulin stimulation.

Work using models has provided further evidence of a molecular link between Aβ and a brain insulin resistant state. In cultured hippocampal neurons, treatment with Aβ oligomers reduces the affinity of insulin binding with its receptor along with promoting removal of insulin receptors from the cell membrane (De Felice et al., 2009; Xie et al., 2002). Furthermore, these changes can be rescued by insulin treatment (De Felice et al., 2009). Aβ oligomers have also been shown to reduce insulin receptor tyrosine autophosphorylation, increase IRS-1 serine phosphorylation, and increase Akt phosphorylation, consistent with changes seen in AD cases (Bomfim et al., 2012; W. Q. Zhao et al., 2008). As mentioned, activation of Akt or its downstream proteins can promote feedback inhibition, through inhibition of the insulin receptor or IRS-1, which may be mediated at least in part by induction of inflammatory signaling by Aβ (Morisco et al., 2005; Ozes et al., 2001; Tian, 2005; Zhang et al., 2008). Transgenic mice with familial AD mutations also show similar insulin signaling changes as seen in AD cases, such as increased IRS-1 serine phosphorylation (Bomfim et al., 2012).

Along with the direct influence of Aβ on the insulin signaling pathway, Aβ and insulin signaling may also interact through an overlapping mechanism of degradation. Several enzymes exist that can degrade Aβ including neprilysin, matrix metalloproteinases (MMP), endothelin-converting enzymes (ECE), angiotensin-converting enzyme (ACE), and insulin degrading enzyme (IDE; also known as insulysin) (Miners et al., 2008). IDE is a zinc metalloendopeptidase that is highly expressed in the liver, testis, muscle and brain and is also the principal enzyme

responsible for insulin degradation (Kuo et al., 1993). In the brain, IDE is primarily expressed in the cytoplasm of neurons, with smaller amounts found in peroxisomes, rough endoplasmic reticulum, and plasma membrane (Miners et al., 2008). IDE deficiency *in vivo* results in accumulation of Aβ in the brain, peripheral hyperinsulinemia, glucose intolerance, and impaired peripheral insulin signaling (Abdul-Hay et al., 2011; Farris et al., 2003). Not surprisingly, reduced IDE levels have been identified in MCI and AD cases (Cook et al., 2003; L. Zhao et al., 2004; Z. Zhao et al., 2007). Furthermore, transgenic AD mice fed high-fat diets show increased Aβ generation that is associated with reduced insulin signaling and IDE in the brain (Ho et al., 2004; L. Zhao et al., 2004) while overexpression of IDE in transgenic AD mice reduces Aβ levels, amyloid plaque burden, and premature death (Leissring et al., 2003). IDE expression is also increased by insulin stimulation (L. Zhao et al., 2004), potentially suggesting that impaired insulin signaling leads to changes in IDE expression/function and thus resulting impairment in Aβ degradation. However, it still remains to be determined how exactly alterations in IDE are related to the insulin signaling changes and Aβ pathology found in AD.

The link between brain insulin resistance and tau pathology in AD, however, is a bit more elusive. It has been previously suggested that insulin signaling changes may induce tau phosphorylation through increased GSK-3β activity, as GSK-3β has been found to phosphorylate tau at multiple sites (Hooper et al., 2008). While some studies have found evidence of increased GSK-3β activation, others have found conflicting results, suggesting that more evidence is required to clearly demonstrate that GSK-3β activity is significantly increased in human AD cases (Griffin et al., 2005; Pei et al., 1997; Steen et al., 2005; Talbot et al., 2012).

Collectively, the above findings point to critical roles of both glutamatergic and insulin signaling in regulation of CNS metabolism and cognitive processes. Not surprisingly, given their

similar functions in the brain, dysfunction of each system has been implicated in AD pathogenesis. However, it has not been previously determined whether glutamatergic signaling dysfunction in AD, particularly through impaired glutamate uptake, is directly related to insulin signaling alterations also identified in the disease. The remainder of this thesis will be aimed at examining the relationship between dysfunction of glutamate uptake and disturbances to CNS insulin signaling in the context of AD.

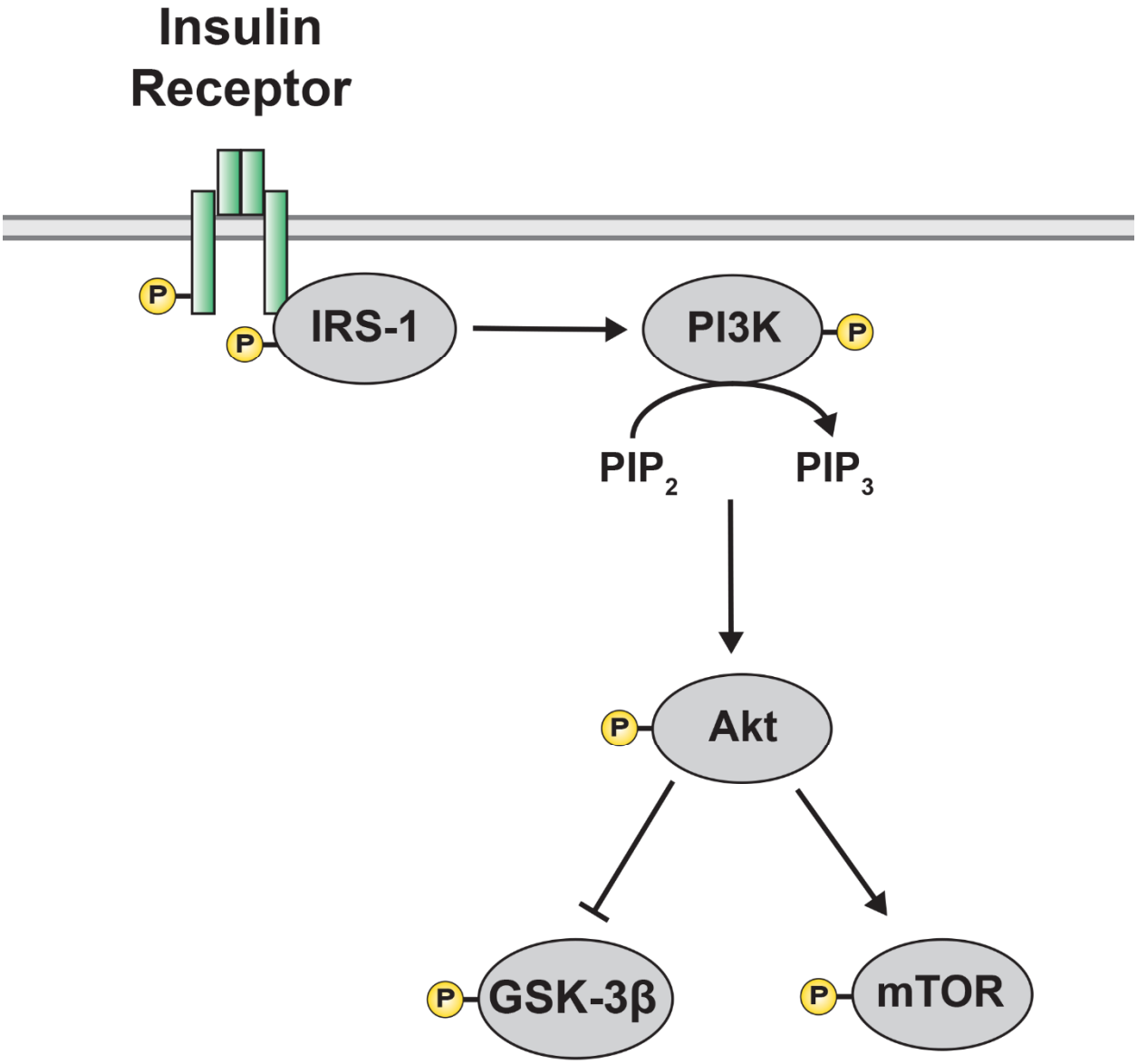

Figure 1.1: The insulin signaling pathway. Activation of the insulin receptor induces a conformational change resulting in autophosphorylation of tyrosine residues along the β-subunit. This promotes recruitment and activation of the insulin receptor substrate-1 (IRS-1) through tyrosine phosphorylation followed by activation of phosphoinositide 3-kinase (PI3K). The catalytic subunit of PI3K then phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP_2) resulting in the formation of PIP_3. This leads to downstream activation of the kinase Akt, which regulates a variety of other proteins including the kinases glycogen synthase kinase-3β (GSK-3β) and mammalian target of rapamycin (mTOR). Akt promotes serine phosphorylation of GSK-3β and mTOR both directly and indirectly, which results in inactivation of GSK-3β and activation of mTOR.

CHAPTER 2:

Partial loss of the glutamate transporter GLT-1 alters brain insulin signaling in a mouse model of Alzheimer's disease[1]

Summary

The glutamate transporter GLT-1 (also called EAAT2 in humans) plays a critical role in regulating extracellular glutamate levels in the CNS. In AD EAAT2 loss is associated with both early and late-stage neuropathology and cognitive impairment. In keeping with this, we have reported that partial GLT-1 loss (GLT-1+/-) causes early-occurring cognitive deficits in mice harboring familial AD APPswe/PS1ΔE9 mutations. GLT-1 plays important roles in molecular pathways that regulate brain metabolism, including insulin and Akt signaling. Significantly, AD pathogenesis also involves chronic Akt activation and reduced insulin signaling in the CNS. In this report we tested the hypothesis that GLT-1 heterozygosity (which reduces GLT-1 to levels that are comparable to losses in AD patients) in APPswe/PS1ΔE9 mice would induce sustained activation of Akt and disturb components of the CNS insulin signaling cascade. We found that partial GLT-1 loss chronically increased Akt activation (reflected by increased phosphorylation at Ser^{473}), impaired insulin signaling (reflected by decreased IRβ phosphorylation of $Tyr^{1150/1151}$ and increased IRS-1 phosphorylation at mouse $Ser^{632/635}$ – denoted as $Ser^{636/639}$ in humans), and reduced insulin degrading enzyme (IDE) activity in brains of mice expressing familial APPswe/PS1ΔE9 AD mutations. GLT-1 loss also caused an apparent compensatory increase in IDE activity in the liver, an organ that has been shown to regulate peripheral Aβ levels and

[1] Chapter 2 reprinted, with minor changes, from the Journal of Alzheimer's Disease, Volume 45(2). Meeker KD, Meabon JS, Cook DG. Partial Loss of the Glutamate Transporter GLT-1 Alters Brain Akt and Insulin Signaling in a Mouse Model of Alzheimer's Disease. Pages 509-20. Copyright 2015, with permission from IOS Press.

shows high expression of GLT-1. Taken together, these findings demonstrate that partial GLT-1 loss can cause insulin signaling abnormalities that are in keeping with those observed in AD.

Introduction

The amino acid glutamate plays a crucial role in excitatory neurotransmission in the CNS. However, once released, extracellular glutamate must be efficiently cleared in order to maintain spatial and temporal resolution of synaptic signaling and to prevent neuronal and synaptic excitotoxicity (Danbolt, 2001; Jarzylo & Man, 2012; Tanaka et al., 1997). As discussed, in the brain this critical function is carried out by a family of five high-affinity Na^+-dependent glutamate transporters referred to as GLAST, GLT-1, EAAC1, EAAT4, and EAAT5 (also known as EAAT1 though EAAT5, respectively). Among these transporters, GLT-1 is especially important in cortex and hippocampus where it is responsible for clearing greater than 90% of the released extracellular glutamate (Danbolt, 2001; Haugeto et al., 1996; Lehre & Danbolt, 1998; Tanaka et al., 1997). The biological significance of GLT-1 is underscored by findings that GLT-1 knockout (-/-) mice develop lethal seizures accompanied by significant hippocampal neuron loss (Tanaka et al., 1997). GLAST, EAAC1, and EAAT4 deficiency are also functionally significant. However, these knockout mice display less severe phenotypes than GLT-1 deficient mice (Aoyama et al., 2006; Huang et al., 2004; Peghini et al., 1997; Watase et al., 1998).

Further supporting the essential role GLT-1 plays in regulating extracellular glutamate levels, loss of EAAT2 (the human homolog of GLT-1) has been reported in patients manifesting a range of neurological diseases or CNS insults including HD (Arzberger et al., 1997; Hassel et al., 2008), TBI (van Landeghem et al., 2006), and ALS (Maragakis et al., 2004; Rothstein et al., 1992; Rothstein et al., 1995). A number of laboratories, including our own, have also recently

reported that EAAT2 is significantly reduced in AD by as much as 50% (Abdul et al., 2009; Jacob et al., 2007; Masliah et al., 1996; Woltjer et al., 2010).

In a previous study we reported that GLT-1 heterozygous ($GLT\text{-}1_{het}$) mice harboring compound familial AD mutations of the amyloid precursor protein (APPswe) and Presenilin 1 (PS1ΔE9) displayed early-occurring cognitive deficits compared to APPswe/PS1ΔE9 mice with normal wild-type GLT-1 ($GLT\text{-}1_{wt}$) levels (Mookherjee et al., 2011). These findings demonstrate that partial GLT-1 loss is capable of unmasking early-occurring cognitive disturbances brought about by expression of the AD-related APPswe/PS1ΔE9 transgene (Mookherjee et al., 2011).

The mechanisms by which GLT-1 loss might contribute to AD-related cognitive disturbances are currently unknown. While there was a significant increase in the ratio of $A\beta_{42}/A\beta_{40}$ in $GLT\text{-}1_{het}$/APPswe/PS1ΔE9 mice, total APP levels and amyloid plaque levels were not affected by GLT-1 heterozygosity (Mookherjee et al., 2011). Thus, amyloid plaque burden appears to be a less likely explanation for the behavioral impairments exhibited by $GLT\text{-}1_{het}$/APPswe/PS1ΔE9 mice.

Similarly, the potential for overt excitotoxicity due to partial GLT-1 loss is also unlikely. In contrast to GLT-1 knockout mice, GLT-1 heterozygous mice appear phenotypically normal (Tanaka et al., 1997). While $A\beta_{42}$ exposure significantly slows the rate at which GLT-1 clears synaptically released glutamate , thereby increasing glutamate spread, we have recently shown that it does not prevent extracellular glutamate concentrations from returning to basal levels (Scimemi et al., 2013) that are estimated to be in the low nanomolar range (Herman & Jahr, 2007). Such findings argue against the idea that the consequences of Aβ-mediated GLT-1 loss are due to chronically elevated basal levels of extracellular glutamate that could induce

excitotoxicity, but potentially rather changes in how long the glutamate can remain in the extracellular space and/or where it is able to act.

In addition to the importance of GLT-1 in protecting against glutamate-induced toxicity (Danbolt, 2001; Jarzylo & Man, 2012; Tanaka et al., 1997), there is evidence that GLT-1 also plays an important role in CNS metabolism. This is supported by data showing that GLT-1 regulates activity-dependent glucose utilization (Herard et al., 2005; Voutsinos-Porche et al., 2003) and GLT-1 expression is regulated by Akt and insulin stimulation (Ji et al., 2011; L. B. Li et al., 2006; Wu et al., 2010). In this regard, a number of studies have reported increased Akt phosphorylation, as well as altered insulin receptor and insulin receptor substrate 1 (IRS-1) expression and function in AD patients (Cholerton et al., 2013; de la Monte & Tong, 2014; Griffin et al., 2005; Pei et al., 2003; Rickle et al., 2004; Talbot et al., 2012).

Such findings prompted us to test the hypothesis that partial GLT-1 loss in APPswe/PS1ΔE9 mice would alter Akt and other insulin signaling proteins *in vivo*. Herein, we report that GLT-1 heterozygosity increased Akt phosphorylation, which was accompanied by alterations in the phosphorylation states of the insulin receptor β-subunit (IRβ) and IRS-1. We also found decreased insulin degrading enzyme (IDE) activity in the CNS. These findings are in keeping with those reported in AD patients and other AD animal models (Cook et al., 2003; Farris et al., 2003; Griffin et al., 2005; Ho et al., 2004; Moloney et al., 2010; Talbot et al., 2012; W. Q. Zhao et al., 2008).

Methods

Animals: APPswe/PS1ΔE9 (APP/PS1) hemizygous mice (line 85) maintained on a B6C3F1/J background (Jankowsky et al., 2004) and GLT-1 heterozygous (GLT_{het}) mice

maintained on a C57BL/6 background (Tanaka et al., 1997) were mated in order to generate F_1 offspring littermates with the genotypes: GLT-1_{wt}/non-transgenic (GLT_{wt}/nTg), GLT-1_{het}/non-transgenic (GLT_{het}/nTg), GLT-1_{wt}/APPswe/PS1ΔE9 (GLT_{wt}/APP/PS1), and GLT-1_{het}/APPswe/PS1ΔE9 (GLT_{het}/APP/PS1). GLT-1 heterozygous mice were a kind gift from Dr. Kohichi Tanaka at Tokyo Medical and Dental University, Tokyo, Japan. Male mice were fed a standard chow diet and given ad libitum access to food and water. All experiments were performed in accordance with procedures approved by the VAPSHCS Institutional Animal Care and Use Committee.

Western blot analysis: Protein lysates were prepared for Western blotting using methods described previously with a few modifications (Mookherjee et al., 2011). Briefly, frozen cortical brain or liver tissue was homogenized in 5 volumes (w/v) of lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0), sonicated, and centrifuged at approximately 20,000 x g for 5 min at 4°C. The pellet and supernatant were divided to prepare crude membrane and soluble fractions, respectively. For the soluble fraction, to analyze phospho-Akt /Akt, phosph-IRS-1/IRS-1, phospho-mTOR/mTOR, phospho-p70 S6 kinase/p70 S6 kinase, and insulin degrading enzyme (IDE), the 20,000 x g supernatant was further centrifuged at approximately 135,000 x g for 1 hour at 4°C. The resulting supernatant was used as the soluble fraction. For the crude membrane fraction, to analyze phospho-IRβ/IRβ, the 20,000 x g pellet was resuspended in lysis buffer containing 1% Triton X-100, briefly sonicated, and centrifuged at 20,000 x g for 30 min. at 4°C. The resulting supernatant was used as the membrane fraction. The lysates were stored at -80°C. Lysate protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Protein lysates were solubilized in Laemmli sample buffer, loaded onto 4-20% SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), electrophoresed, and transferred onto PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in 5% (w/v) non-fat dried milk dissolved in Tris-buffered saline (20 mM Tris, 140 mM NaCl, pH 7.4) with 0.03% Tween-20 and subsequently incubated with specific primary antibodies against phospho-insulin receptor β-subunit (pIRβ, $Tyr^{1150/1151}$), total insulin receptor β-subunit (IRβ), phospho-Akt (pAkt, Ser^{473}), total Akt, phospho-IRS-1 (pIRS-1, detects $Ser^{632/635}$ in mice and $Ser^{636/639}$ in humans), total IRS-1, phospho-mTOR (p-mTOR, Ser^{2448}), total mTOR, phospho-p70 S6 kinase (p-p70 S6K, Thr^{389}), total p70 S6 kinase, IDE, or pyruvate kinase overnight at 4°C. All primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) with the exceptions of pan-specific antibodies to IRβ and IDE, which were obtained from Millipore (Billerica, MA), and anti-pyruvate kinase that was obtained from Rockland Immunochemicals (Gilbertsville, PA). Western blots were then incubated in the corresponding horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and were developed using Amersham enhanced chemiluminescence (ECL) (GE Healthcare, Piscataway, NJ) and an ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ). Densitometry analyses were performed using ImageQuant TL software (GE Healthcare, Piscataway, NJ). Band intensities of phosphorylated proteins were normalized to band intensities of corresponding total protein levels. For each protein examined (Akt, IRβ, mTOR, p70 S6K, and IRS-1) the ratios of phospho- epitope / total protein were normalized to the GLT_{wt}/nTg group as 100%.

IDE activity: The degradation of insulin was measured using a trichloroacetic acid (TCA) solubility assay in accordance with previously established methods (Bennett et al., 2000;

Farris et al., 2003). Briefly, bovine serum albumin was added to siliconized tubes to prevent adsorption of the substrate. Soluble fraction protein lysates (20 μg) of brain (cortex) or liver were then incubated with [^{125}I]-insulin (Phoenix Pharmaceuticals, Inc., Burlingame, CA) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 90 min. (brain) or 45 min. (liver) at 22 °C (reaction volume 100 μl). The reaction was terminated by adding 300 μl of cold 13.3% trichloroacetic acid. After chilling for 15 min at 4°C, the samples were centrifuged at approximately 15,000 x g for 10 min, 200 μl of supernatant was removed and the radioactivity was counted using a gamma counter (Model 41600 HE; ICN Biomedicals, Costa Mesa, CA). To obtain a baseline level of degradation, negative controls were obtained for each sample by adding a 10,000-fold excess of non-radiolabeled insulin. For degradation counts, the negative control count was subtracted from the sample count and the activity data for each genotype was normalized to the GLT_{wt}/nTg group.

Statistics: Data are presented as means ± standard error of the mean (SEM) and analyzed using standard analysis of variance (ANOVA) methods. Planned comparison Helmert tests (Field, 2005; Winer et al., 1991) were performed upon a statistically significant ANOVA result ($p \leq 0.05$) to test the *a priori* hypothesis that wild-type, non-transgenic mice would be different from GLT-1 heterozygous and transgenic APPswe/PS1ΔE9 mice. Statistical analyses were carried out using SPSS software (IBM, Armonk, NY).

Results

Sustained Akt activation, as reflected by increased levels of phosphorylation at Ser^{473}, has been reported in brains of AD patients (Griffin et al., 2005; Pei et al., 2003; Rickle et al., 2004; Talbot et al., 2012). To examine whether partial GLT-1 loss in the context of AD-related

pathology alters CNS Akt signaling *in vivo*, we measured phospho-Akt (Ser^{473}) and total Akt levels in immunoblots of protein lysates prepared from cortex of a cohort of mice comprised of four experimental groups: (*i*) GLT_{wt}/nTg; (*ii*) GLT_{het}/nTg; (*iii*) GLT_{wt}/APP/PS1; and (*iv*) GLT_{het}/APP/PS1. Previously we reported that GLT-1 heterozygosity caused significant behavioral impairments that were more prominent at 6 months than at 9 months of age; and which corresponded to a significant increase in the ratio of $A\beta_{42}/A\beta_{40}$ at 6, but not at 9 months of age (Mookherjee et al., 2011).

GLT-1 heterozygosity and APP/PS1 transgene expression activate Akt:

At 6 months (Figure 2.1A and 2.1C) we found a statistically significant overall difference among genotypes in the ratios of phospho-Akt (Ser^{473}) to total Akt protein ($F[3,18]=5.103$, $p \leq 0.01$). A Helmert specific comparisons test (Field, 2005; Winer et al., 1991) confirmed that phospho-Akt/Akt ratios were significantly lower in GLT_{wt}/nTg mice compared to the other three groups ($p < 0.003$). Similarly, at 9 months (Figure 2.1B and 2.1D) there was a statistically significant overall difference among genotypes in phospho-Akt/Akt ratios ($F[3,20]=9.787$, $p < 0.001$). Among the 9 month cohort, Helmert analysis revealed that phospho-Akt/Akt ratios were significantly lower in GLT_{wt}/nTg mice compared to the other three groups ($p < 0.003$). However, at 9 months this difference in Akt phosphorylation among the GLT_{het}/nTg and GLT_{wt}/APP/PS1 groups was not as pronounced as at 6 months (Figures 2.1B and 2.1D).

GLT-1 loss or APP/PS1 transgene expression suppresses insulin receptor activation:

The findings in Figure 2.1 demonstrate that GLT-1 heterozygosity and APPswe/PS1ΔE9 transgene expression caused prolonged activation of Akt. It has been reported that sustained Akt activation can lead to inhibition of insulin receptor autophosphorylation through negative feedback regulation (Morisco et al., 2005; Tian, 2005; W. Q. Zhao et al., 2008). In order to test

whether sustained Akt elevation due to GLT-1 heterozygosity and/or APP/PS1 expression would be accompanied by reduced insulin receptor phosphorylation, we measured the levels of phosphorylated insulin receptor β-subunit (IRβ-$Tyr^{1150/1151}$) and total IRβ by Western blot analysis.

At 6 months (Figure 2.2A and 2.2C) we found an overall difference among genotypes in the ratio of phospho-IRβ ($Tyr^{1150/1151}$) to total IRβ protein ($F[3,20]=3.374$, $p \leq 0.039$). A Helmert analysis supported the conclusion that both GLT-1 heterozygosity and APP/PS1 transgene expression were sufficient to significantly suppress IRβ phosphorylation in all three groups compared to GLT_{wt}/nTg mice ($p \leq 0.005$). However, at 9 months (Figure 2.2B and 2.2D) there was no statistically significant difference among groups in the ratios of phospho-IRβ ($Tyr^{1150/1151}$)/IRβ ($F[3,20]=2.042$, n.s.). These findings indicate that at 6 months (a time point at which behavioral and $A\beta_{42}/A\beta_{40}$ abnormalities were most prominent (Mookherjee et al., 2011)), partial GLT-1 loss or APP/PS1 transgene expression decreased tyrosine phosphorylation of the insulin receptor.

GLT-1 heterozygosity alters IRS-1, but not mTOR signaling:

The insulin receptor substrate-1 (IRS-1) is a critical adapter protein that is responsible for transmitting signals from the insulin receptor to intracellular pathways (Taniguchi et al., 2006). IRS-1 is a key target for negative feedback regulation of insulin signaling (Boura-Halfon & Zick, 2009; Hotamisligil et al., 1996) and there is evidence that Akt activation may be in part responsible for regulation of IRS-1 (Ozes et al., 2001).

At 6 months (Figure 2.3A and 2.3C) we found a statistically significant difference among the four groups in the ratio of phospho-IRS-1($Ser^{632/635}$)/IRS-1 ($F[3,18]= 3.885$, $p \leq 0.027$). Phospho-IRS-1/IRS-1 ratios were significantly elevated by GLT-1 heterozygosity, whether or

not the animals expressed an APP/PS1 transgene (GLT-1: F[1,18]=10.183, $p \leq 0.005$; APP: F[1,18]=0.296, n.s.). While there appeared to be a similar trend of elevated IRS-1 $Ser^{632/635}$ phosphorylation among the GLT-1_{het} animals (Figure 2.3B and 2.3D), phospho-IRS-1/IRS-1 ratios in the 9 month-old animals were not statistically significant (F[3, 20]= 2.445, n.s.) and neither was the outcome of a two-factor analysis of GLT-1 genotype and APP/PS1 transgene status (GLT-1: F[1,20]=3.442, n.s.; APP: F[1,20]=3.486, n.s.).

Negative feedback regulation of IRS-1 after prolonged Akt activation has been suggested to occur through mammalian target of rapamycin (mTOR), a downstream target of Akt (Ozes et al., 2001). To address this possibility we assessed mTOR activity by measuring phosphorylation at Ser^{2448}. For further confirmation of any changes in mTOR activity, we also measured phosphorylation of p70 S6 kinase (p70 S6K-Thr^{389}), a downstream target of mTOR often used as an indicator of mTOR signaling (Burnett et al., 1998; Magnuson et al., 2012). We found no statistically significant changes in the ratios of phospho-mTOR (Ser^{2448}) to total mTOR or phospho-p70 S6K (Thr^{389}) to total p70 S6K at either 6 months (**mTOR**: means +/- SEM and (N) = 1.00 +/- 0.13 (6), 1.14 +/- 0.40 (5); 1.67 +/- 0.26 (6), 1.24 +/- 0.12 (5); F[3,18]=1.469, n.s.; **p70 S6K**: 1.00 +/- 0.25 (6), 0.59 +/- 0.14 (5), 0.53 +/- 0.12 (6), 0.65 +/- 0.08 (5); F[3,18]=1.649, n.s.; GLT_{wt}/nTg, GLT_{het}/nTg, GLT_{wt}/APP/PS1, and GLT_{het}/APP/PS1, respectively) or at 9 months of age (**mTOR**: means +/- SEM and (N) = 1.00 +/- 0.34 (6), 0.94 +/- 0.27 (6); 1.05 +/- 0.36 (6), 1.53 +/- 0.41 (6); F[3,20]=0.618, n.s. **p70 S6K**: 1.00 +/- 0.50 (6), 1.50 +/- 0.49 (6), 1.05 +/- 0.27 (6), 2.05 +/- 0.71 (6); F[3,20]=0.897, n.s.; GLT_{wt}/nTg, GLT_{het}/nTg, GLT_{wt}/APP/PS1, and GLT_{het}/APP/PS1, respectively). These findings argue against the idea that partial GLT-1 loss influenced mTOR signaling.

GLT-1 regulates IDE activity:

Brain insulin signaling changes that are in general keeping with those reported herein have been argued to underlie reduced insulin degrading enzyme (IDE) expression in AD, which also degrades Aβ (Cholerton et al., 2013; Cook et al., 2003; Farris et al., 2003; Miners et al., 2008; Z. Zhao et al., 2007). In addition, decreased phosphorylation of IRβ ($Tyr^{1150/1151}$) is also associated with reduced IDE activity in mice with impaired insulin signaling due to a high fat diet (Ho et al., 2004). In order to address whether GLT-1 heterozygosity and/or APP/PS1 transgene expression alters IDE activity in the brain, we measured the levels of trichloroacetic acid (TCA)-soluble counts after incubation of cortical protein lysates with [^{125}I]-insulin. These counts were normalized to counts obtained from samples incubated with excess non-radiolabeled insulin. Table 2.1 shows that partial loss of GLT-1, but not APP/PS1 transgene expression, significantly reduced IDE activity and that IDE activity levels did not differ significantly between 6 and 9 month old mice (GLT-1: $F[1,40]=6.930$, $p \leq 0.012$; APP: $F[1,40]=0.704$, n.s.; Age: $F[1,40]= 0.902$, n.s., respectively). Table 2.1 also shows that IDE protein levels measured by Western blot were not statistically different at either 6 or 9 months of age (GLT-1: $F[1,38]=2.030$, n.s.; APP: $F[1,38]=0.370$, n.s.: Age, $F[1,38]=2.029$, n.s.).

These results indicate that partial GLT-1 loss significantly reduced the enzymatic activity of cortical IDE, an enzyme that has been found to be reduced in AD (Cook et al., 2003; Miners et al., 2008), even at early stages of the disease (Z. Zhao et al., 2007). This also raises an interesting question as to why overall amyloid levels were not elevated in these mice (Mookherjee et al., 2011) in that IDE degrades both $A\beta_{40}$ and $A\beta_{42}$ (Mukherjee et al., 2000). While there are multiple proteases capable of catabolizing Aβ (Tanzi et al., 2004), another robust means of clearing CNS Aβ involves Aβ transiting the blood-brain barrier into the peripheral circulation followed by rapid degradation in the liver (Ghiso et al., 2004; Marques et al., 2009; Zlokovic,

2004, 2008). Both IDE and GLT-1 are highly expressed in liver (Berger & Hediger, 2006; Duckworth et al., 1998). In addition, the liver has been shown to regulate Aβ clearance from the brain by affecting circulating peripheral Aβ levels (Marques et al., 2009).

To determine whether partial GLT-1 loss influenced IDE activity in the liver we measured trichloroacetic acid (TCA)-soluble counts after incubation of liver protein lysates with [^{125}I]-insulin. Again, these counts were normalized to counts obtained from samples incubated with excess non-radiolabeled insulin. Table 2.2 shows that partial GLT-1 loss, but not expression of the APP/PS1 transgene, caused a marked, significant increase in IDE activity in the liver (GLT-1: $F[1,36]=42.717$, $p<0.001$; APP: $F[1,36]=3.457$, n.s.; Age: $F[1,36]=1.882$, n.s.). Liver IDE protein levels were not significantly different with respect to genotype factor (GLT-1: $F[1,36]=1.103$, n.s.; APP: $F[1,36]=0.252$, n.s.), but were overall higher in the older mice (Age: $F[1,36]=5.115$, $p\leq 0.030$).

These results provide additional evidence that GLT-1 regulates IDE activity. Moreover, they also suggest the possibility that elevated IDE activity in the liver could compensate for impaired CNS IDE activity by facilitating systemic Aβ clearance (see Discussion).

Discussion

Glutamate is the primary excitatory neurotransmitter in the brain and is essential for normal CNS activity. The significance of glutamatergic signaling is underscored by estimates that as much as 80% of the metabolic energy consumed by the CNS is related to glutamate cycling (Raichle & Gusnard, 2002). This indicates that even at rest, glutamate cycling places a sustained metabolic burden on brain cells that require highly efficient and dynamic regulatory systems in order to prevent glutamate-induced CNS dysfunction. In keeping with the critical importance of rapidly clearing this amino acid, glutamate transporters are highly expressed in the

brain where, for example, GLT-1 represents approximately 1% of the total tissue protein in hippocampal CA1 stratum radiatum (Lehre & Danbolt, 1998).

Involvement of GLT-1 in metabolically significant brain functions:

A growing body of evidence indicates that GLT-1 plays important roles in regulating pathways that govern the metabolic status of the CNS. Modulation of activity-dependent glucose utilization is regulated by both GLT-1 and GLAST where it has been shown that partial glutamate transporter loss can impair glutamate-evoked, but not basal glucose uptake in astrocytes (Voutsinos-Porche et al., 2003). Also, recent findings show that synaptic activity regulates co-localization of mitochondria with GLT-1 at sites of glutamate uptake (Jackson et al., 2014). Significantly, a number of studies show that treatments which activate the IR/IRS-1/Akt signaling cascade (Sesti, 2006; Talbot et al., 2012) can regulate GLT-1 expression levels (Ji et al., 2011; L. B. Li et al., 2006; Wu et al., 2010). For example, Li and colleagues (L. B. Li et al., 2006) have reported that viral vectors expressing constitutively active Akt transgenes increase GLT-1 expression in primary cultured astrocytes. Interestingly, in this latter example altering Akt activity modulated GLT-1 expression, whereas in our experiments Akt signaling was altered by GLT-1 heterozygosity. This apparent distinction is likely due to differences between *in vitro* and *in vivo* experimental conditions, where GLT-1 expressing cells interact within synaptic networks, neurovascular elements, and other CNS systems in more complex ways than can reasonably be approached with isolated astrocytes in culture. Furthermore, changes in insulin signaling in our model could be representative of neuronal alterations, rather than astrocytic, as insulin signaling molecules show higher expression in neurons. Insulin signaling changes in AD have also been more prominently identified in neurons as well (Griffin et al., 2005; Moloney et al., 2010; Talbot et al., 2012). More importantly, both the *in vitro* (L. B. Li et al., 2006) data and

our *in vivo* findings independently demonstrate that Akt and GLT-1 interact functionally in a significant metabolic pathway that likely accommodates both feed-forward and feedback responses evoked by glutamatergic signals.

GLT-1 is expressed at low levels in the pancreas (Meabon et al., 2012), thereby raising the question whether some aspects of GLT-1 function might be related to pancreatic GLT-1 endocrine and/or exocrine activity. However, recent studies using mice with a brain-specific GLT-1 deficiency demonstrate that GLT-1 loss in the brain alone is sufficient to account for the primary phenotypes associated with total systemic GLT-1 loss (Tanaka et al., 1997; Y. Zhou et al., 2014). Moreover, GLT-1 expression levels in the pancreas are considered too low to be functionally significant in clearing extracellular pancreatic glutamate (Y. Zhou et al., 2014). The animals used in this study were committed to biochemical analyses before we appreciated there may be GLT-1 mediated changes in insulin signaling. Therefore, we do not know whether GLT-1 heterozygous animals displayed peripheral insulin resistance. However, this possibility is unlikely as the mice were maintained on normal mouse chow, and more importantly, there were no significant differences in body weight among the genotype groups (Mookherjee et al., 2011).

Involvement of GLT-1 in AD-related pathology:

At 6 months of age, but not at 9 months, the mice studied herein exhibited a significant increase in the ratio of $A\beta_{42}/A\beta_{40}$ among GLT-1 heterozygotes compared to animals with normal, wild-type GLT-1 levels (Mookherjee et al., 2011). While this increase was modest, this raises the possibility that $A\beta_{42}$ levels might have contributed to the overall pattern of altered Akt, IRβ, and IRS-1 phosphorylation, which collectively was more pronounced in the 6 month animals than in the 9 month-old mice. Other investigators have reported that exposing primary cultured neurons to oligomeric $A\beta_{42}$ (also known as amyloid-β-derived diffusible ligands, or

ADDLs) increases phospho-Akt (Ser^{473}) with an accompanying reduction in phospho-IRβ ($Tyr^{1150/1151}$) levels (W. Q. Zhao et al., 2008). In addition, glutamate exposure (simulating synaptic glutamate release) and treatments that stimulate neuronal glutamate release also reduced phospho-IRβ ($Tyr^{1150/1151}$) (W. Q. Zhao et al., 2008). These data are consistent with our findings which show that both partial GLT-1 loss and expression of an APPswe/PS1ΔE9 transgene increases Akt (Ser^{473}) phosphorylation; and is associated with a similar reduction in IRβ ($Tyr^{1150/1151}$) phosphorylation.

We and others have reported that GLT-1 is significantly reduced and aberrantly expressed in both mild cognitively impaired and late-stage AD patients (Abdul et al., 2009; Jacob et al., 2007; Masliah et al., 1996; Woltjer et al., 2010). Such losses would not be expected to prevent restoration of basal extracellular glutamate levels following synaptic glutamate release (Diamond & Jahr, 2000; Herman & Jahr, 2007). However, partial glutamate transporter loss has a significant impact on the spread of synaptically released glutamate that can degrade pathway specificity of synaptic transmission and plasticity (Diamond & Jahr, 1997; Tsvetkov et al., 2004). In keeping with this, we have recently reported that exposing hippocampal slices to oligomeric $A\beta_{42}$ reduces GLT-1 activity, thereby approximately doubling the time required to clear endogenous, synaptically released glutamate by specifically reducing GLT-1 activity (Scimemi et al., 2013).

We found that partial GLT-1 loss, but not APP/PS1 mutant transgene expression, reduced IDE activity levels in the brain (Table 2.1). Other studies have shown that reduced CNS IDE activity and protein levels are associated with impaired insulin signaling and are also associated with AD pathology in humans and animal models of AD (Abdul-Hay et al., 2011; Cholerton et al., 2013; Cook et al., 2003; Farris et al., 2003; Leissring et al., 2003; Miners et al., 2008; Z.

Zhao et al., 2007). Whether GLT-1-specific reductions in IDE activity are driven by changes in Akt, IRβ, or IRS-1, or whether reduced IDE activity might cause the observed insulin signaling changes, cannot be discerned from the data reported herein. The finding that partial GLT-1 loss reduces IDE activity provides additional independent evidence supporting the conclusion that insulin-related disturbances are caused by GLT-1 loss.

In conjunction with reduced brain IDE activity, GLT-1 loss also caused a marked increase in IDE activity in the liver, which expresses high levels of GLT-1in zone 3 hepatocytes (Cadoret et al., 2002; Meabon et al., 2012). While we found a significant increase in the ratio of $A\beta_{42}/A\beta_{40}$ in these mice at 6 months of age, we did not find any other significant changes in Aβ accumulation between the GLT_{wt}/APP/PS1 and GLT_{het}/APP/PS1 groups at either 6 or 9 months (Mookherjee et al., 2011). CNS Aβ is efficiently cleared into the peripheral circulatory system, which is often referred to as a peripheral Aβ sink (Brody & Holtzman, 2008; Zlokovic, 2004) . We and others have shown that the liver functions as a critical systemic Aβ drain (Ghiso et al., 2004; Hone et al., 2003; Marques et al., 2009; Tamaki et al., 2006). Importantly, peripheral Aβ clearance by the liver can regulate CNS Aβ clearance (Marques et al., 2009). Thus, it is possible that the increased hepatic IDE activity associated with GLT-1 heterozygosity could potentially help compensate for decreased IDE activity in the brain by increasing the dynamics of peripheral Aβ clearance mediated by the liver.

GLT-1 loss as an early factor in AD pathology:

We found that partial GLT-1 loss had more pronounced effects on Akt, IRβ, and IRS-1 signaling at 6 months than at 9 months of age. This outcome is consistent with the behavioral impairments manifested by these mice (Mookherjee et al., 2011), thereby strengthening the idea

that GLT-1 loss can drive cognitive impairments via mechanisms that involve early-occurring insulin signaling defects.

Overall, these findings are in keeping with the idea that GLT-1 loss primarily influences AD-related neuropathology early in the pathogenic process, prior to later time points when amyloid deposition becomes a more dominant pathological factor in these APP/PS1 transgenic mice that accumulate Aβ at higher rates after 6 months (Jankowsky et al., 2004).

These data linking GLT-1 loss to insulin signaling defects correspond comparatively well with findings in AD patients. Nonetheless, a limitation of this animal model is that in AD GLT-1 loss in the brain is progressive over the course of disease (Woltjer et al., 2010) and in humans, presumably normal adult levels of GLT-1 are maintained for years prior to the onset of latent pathogenic processes. In the mice, genetically-mediated GLT-1 losses occur in a stepwise fashion where GLT-1 heterozygous mice express 50-60% of the GLT-1 from birth. It is possible this could contribute to complex interactions between GLT-1 heterozygosity and prolonged APP/PS1 transgene expression that could also influence the prominence of GLT-1 loss on cognition and insulin signaling at earlier, rather than later time points.

The findings presented in this chapter demonstrate that partial GLT-1 loss causes sustained Akt activation *in vivo* that is associated with feedback-related impairment of insulin signaling; and which together mimic the pattern of cognitive impairments in these mice. These results are consistent with prior findings that implicate Akt activity and insulin signaling in GLT-1 regulation. Taken collectively, these studies expand and strengthen the argument that GLT-1 plays an important role in regulating the metabolic status of the brain. In addition, these results suggest the possibility that GLT-1 dysfunction/loss which attends AD pathogenesis may be a

contributing mechanism to the disturbances in the insulin signaling pathway that have been identified in AD (Cholerton et al., 2013; de la Monte & Tong, 2014; Talbot et al., 2012).

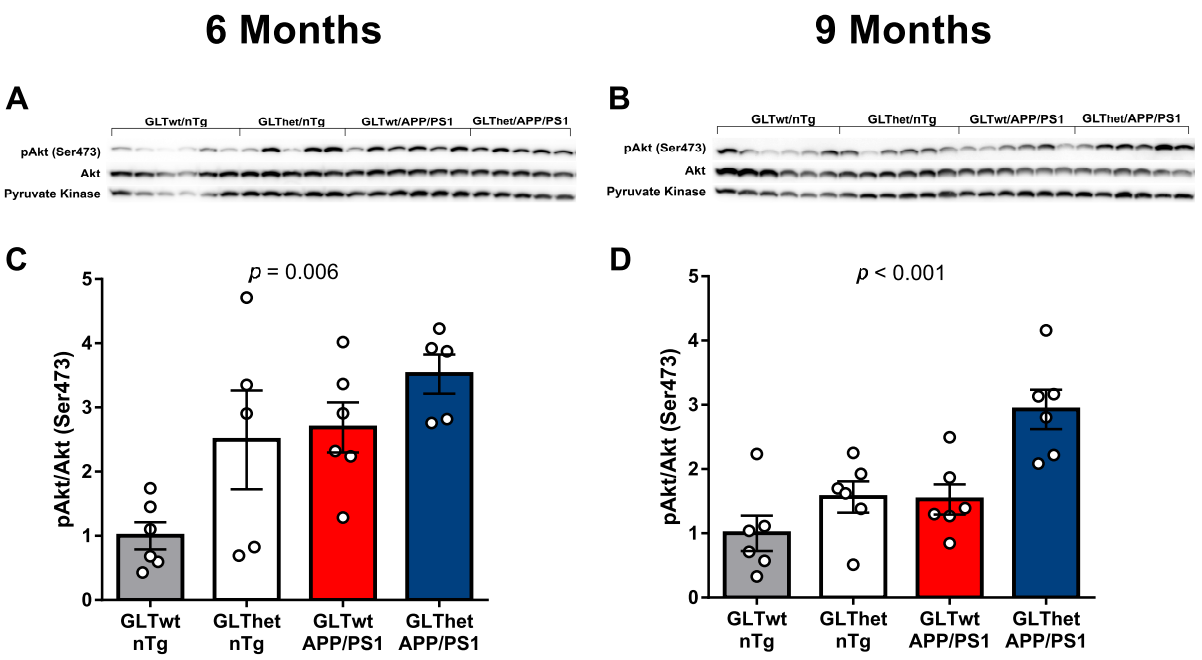

Figure 2.1: Akt phosphorylation is increased by partial loss of GLT-1 and expression of the APP/PS1 transgene and is more pronounced at 6 months of age. (A and B) Western blots from 6 and 9 month animals, respectively. (C and D) Indicating sustained activation of Akt, densitometric quantification of blots in panels A and B revealed a statistically significant increase in the ratio of phosphorylated Akt (Ser^{473}) to total Akt in GLT_{het} and APP/PS1 transgene mice compared to $GLT\text{-}1_{wt}$/nTg mice at both 6 (C) and 9 (D) months of age ($p \leq 0.01$ and $p \leq 0.001$, respectively). Each lane represents one animal. Data are presented as normalized means ± SEM (n = 5-6 mice per group).

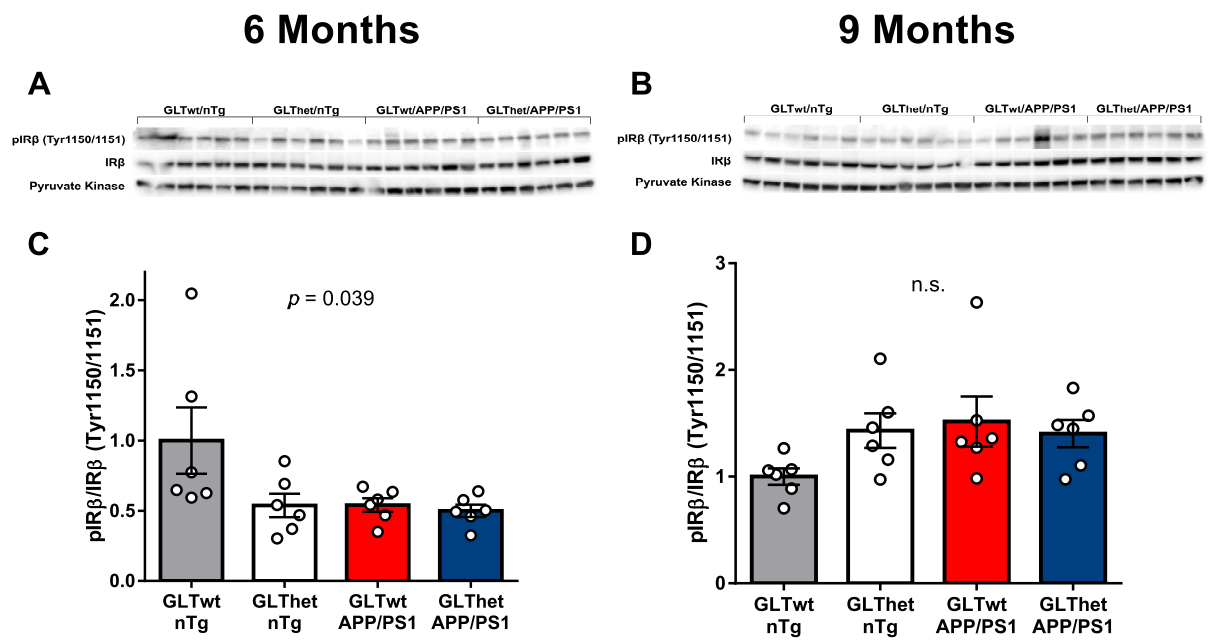

Figure 2.2: Insulin receptor autophosphorylation is reduced by partial loss of GLT-1 and APP/PS1 at 6 months of age. (A and B) Western blots from 6 and 9 month animals, respectively. (C and D) Indicating reduced insulin receptor activation, densitometric quantification of blots in panels A and B revealed a statistically significant decrease ($p \leq 0.039$) in the ratio of $Tyr^{1150/1151}$ phosphorylated IRβ to total IRβ in GLT_{het} and APP/PS1 transgene mice compared to GLT-1_{wt}/nTg mice at 6 (C), but not at 9 (D) months of age. Each lane represents one animal. Data are presented as normalized means ± SEM (n = 6 mice per group).

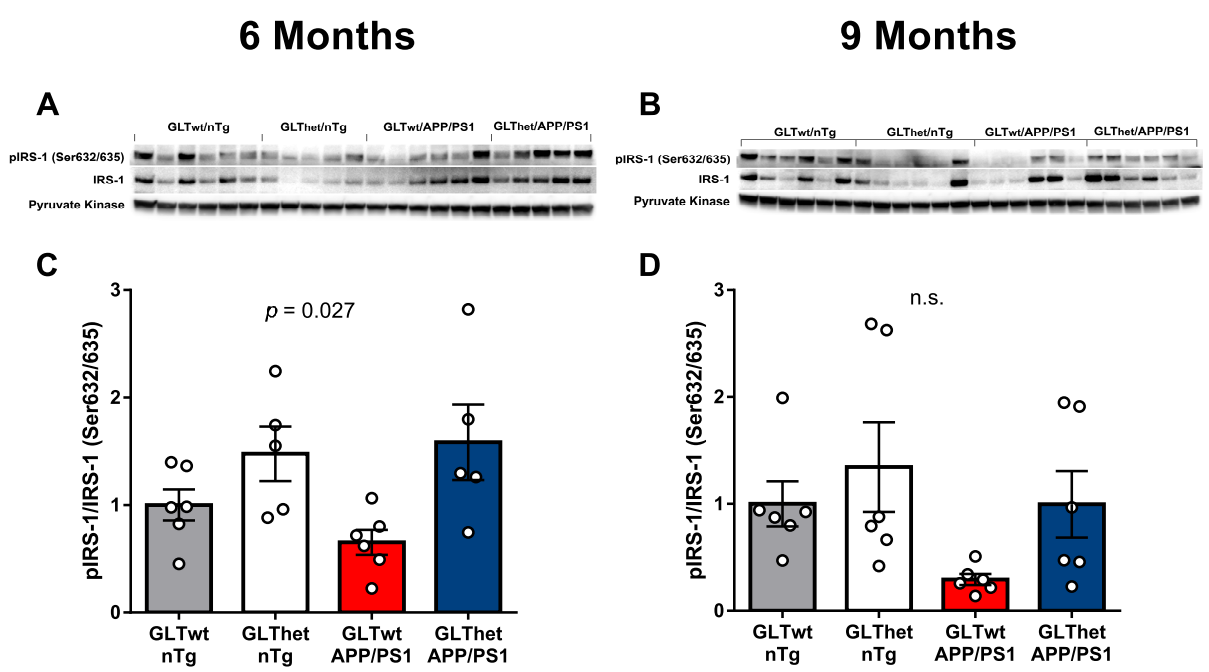

Figure 2.3: IRS-1 serine phosphorylation is increased by partial loss of GLT-1 at 6 months of age. (A and B) Western blots from 6 and 9 month animals, respectively. (C and D) Indicating reduced IRS-1 signaling, densitometric quantification of blots in panels A and B revealed a statistically significant increase ($p \leq 0.027$) in the ratio of Ser632/635 phosphorylated IRS-1 to total IRS-1 in mice with partial loss of GLT-1, but not animals with an APP/PS1 transgene at 6 months of age (C). Phospho-IRS-1/IRS (Ser632/635) ratios were not significantly different in 9 month mice (D). Each lane represents one animal. Data are presented as normalized mean ± SEM (n = 5-6 mice per group).

Table 2.1: In the brain, partial loss of GLT-1 reduces IDE activity, but not IDE protein expression.

Group	IDE Activity[a]			IDE Protein Expression[b]		
	6 Months	9 Months		6 Months	9 Months	
GLT_{wt}/nTg	1.00 ± 0.08	1.00 ± 0.10	$p < 0.012$ *(GLT-1 +/- vs GLT-1 +/+)*	1.00 ± 0.04	1.00 ± 0.08	n.s. *(GLT-1 +/- vs GLT-1 +/+)*
GLT_{wt}/APP/PS1	1.04 ± 0.09	0.87 ± 0.06		0.86 ± 0.08	0.94 ± 0.06	
GLT_{het}/nTg	0.82 ± 0.07	0.86 ± 0.03		1.00 ± 0.01	1.01 ± 0.08	
GLT_{het}/APP/PS1	0.85 ± 0.17	0.71 ± 0.06		1.01 ± 0.13	1.07 ± 0.08	

[a] IDE activity was measured by the level of trichloroacetic acid-soluble counts after incubation of lysates with [^{125}I]-insulin and were normalized to samples incubated with excess non-radiolabeled insulin. Data was then normalized to the mean of the GLT_{wt}/nTg group defined as 1. A three-way analysis of variance (ANOVA) revealed that partial GLT-1 (GLT-1 +/+ vs. GLT-1 +/-) loss caused a significant decrease in IDE activity ($p < 0.012$), while the effects of APP/PS1 transgene expression (APP/PS1 vs non-transgenic) and age (6 vs 9 months) were not statistically significant. Data are presented as mean ± standard error of the mean (SEM). n = 6 mice per group.

[b] IDE protein expression was measured by Western blot densitometry and normalized with respect to actin immunoreactivity reprobed on the same blot. As with IDE activity, IDE protein levels were normalized with respect to the mean of the GLT_{wt}/nTg group defined as 1. A three way ANOVA revealed no statistically differences for any factors (GLT-1 genotype, APP/PS1 expression, or age). Data are presented as mean ± standard error of the mean (SEM). n = 5-6 mice per group.

Table 2.2: In the liver, partial loss of GLT-1 increases IDE activity, but not IDE protein expression.

Group	IDE Activity[a]			IDE Protein Expression[b]		
	6 Months	9 Months		6 Months	9 Months	
GLT_{wt}/nTg	1.00 ± 0.28	1.00 ± 0.95	$p < 0.001$ *(GLT-1 +/- vs GLT-1 +/+)*	1.00 ± 0.10	1.00 ± 0.03	n.s. *(GLT-1 +/- vs GLT-1 +/+)*
GLT_{wt}/APP/PS1	0.49 ± 0.08	0.86 ± 0.25		1.11 ± 0.17	1.38 ± 0.09	
GLT_{het}/nTg	4.14 ± 0.72	2.08 ± 0.22		1.04 ± 0.15	1.14 ± 0.09	
GLT_{het}/APP/PS1	3.10 ± 0.44	1.65 ± 0.21		0.70 ± 0.12	1.20 ± 0.05	

[a] IDE activity was measured by the level of trichloroacetic acid-soluble counts after incubation of lysates with [^{125}I]-insulin and were normalized to samples incubated with excess non-radiolabeled insulin. Data was then normalized to the mean of the GLT_{wt}/nTg group defined as 1. A three-way analysis of variance (ANOVA) revealed that partial GLT-1 (GLT-1 +/+ vs. GLT-1 +/-) loss caused a significant increase in IDE activity ($p < 0.001$), while the effects of APP/PS1 transgene expression (APP/PS1 vs non-transgenic) and age (6 vs 9 months) were not statistically significant. Data are presented as mean ± standard error of the mean (SEM). n = 2-9 mice per group.

[b] IDE protein expression was measured by Western blot densitometry and normalized with respect to pyruvate kinase immunoreactivity reprobed on the same blot. As with IDE activity, IDE protein levels were normalized with respect to the mean of the GLT_{wt}/nTg group defined as 1. A three-way ANOVA (GLT-1 genotype, APP/PS1 expression, and age) revealed a statistically significant difference between the two ages. Upon further analysis, a two-way ANOVA (GLT-1 genotype and APP/PS1 expression) of 6 month data revealed no significant differences while at 9 months there was only a significant effect of APP/PS1 expression ($p < 0.019$). Data are presented as mean ± standard error of the mean (SEM). n = 2-9 mice per group.

CHAPTER 3:

Attenuation of insulin signaling by loss of GLT-1 function in neurons is dependent on NMDA receptor activation

Summary

In the previous chapter, I showed that partial loss of GLT-1 in a mouse model of AD results in disturbances to insulin signaling in the brain, a pathway that is known to regulate cognitive processes and is also altered in AD, which were associated with early-occurring cognitive deficits previously identified in these mice. However, the mechanism for how GLT-1 loss leads to alterations in insulin signaling was not identified. In conjunction with astrocytic expression, GLT-1 is also expressed in neuronal axon terminals where it has been found to be responsible for a significant portion of total uptake, yet its function there is unknown. Insulin signaling disturbances in AD have also been primarily identified in neurons. Thus, I hypothesized that partial GLT-1 loss in neurons may contribute to the overall pattern of altered insulin signaling reported in Chapter 2. To specifically address this hypothesis, I utilized primary cortical neurons to examine the effects of GLT-1 inhibition on insulin signaling. Inhibition of GLT-1 resulted in blunted insulin-evoked phosphorylation of the insulin receptor and decreased total IRS-1 levels. Furthermore, GLT-1 inhibition also resulted in reduced insulin-evoked and basal phosphorylation levels of downstream insulin signaling molecules including Akt, GSK-3β, and mTOR. The insulin signaling changes induced by GLT-1 inhibition were prevented by scavenging of extracellular glutamate and NMDA receptor inhibition, possibly those localized to extrasynaptic sites. Collectively, I show that loss of neuronal GLT-1 function results in glutamatergic signaling dyshomeostasis and disturbed insulin signaling. Furthermore, inhibition

of both neuronal glutamate transporters, GLT-1 and EAAC1, produced similar changes but to a slightly greater extent suggesting similar, cooperative functions in regulation of glutamatergic and insulin signaling.

Introduction

After release into the synapse, extracellular glutamate concentration needs to be tightly regulated to maintain spatial and temporal resolution of signaling and to prevent excitotoxicity due to overstimulation of glutamatergic receptors (Danbolt, 2001; Jarzylo & Man, 2012; Tanaka et al., 1997). As discussed earlier, GLT-1 is the most abundant glutamate transporter in the CNS and has been found to be responsible for the vast majority of glutamate uptake in the forebrain (Danbolt, 2001; Haugeto et al., 1996; Lehre & Danbolt, 1998; Tanaka et al., 1997). In these regions, such as the cortex and hippocampus, GLT-1 expression is highest in astrocytes, but roughly 10% of GLT-1 expression has been identified in neuronal glutamatergic axon terminals (Furness et al., 2008). Despite this substantial difference in expression levels, however, uptake of substrate was found to be as fast in axon terminals as in astrocytes (Furness et al., 2008). Furthermore, a recent study found that neuronal GLT-1 is responsible for a much larger percentage of synaptosomal glutamate uptake compared to astrocytic GLT-1 (Petr et al., 2015). However, there is currently a limited understanding of the physiological function of neuronal GLT-1.

Dysfunction of GLT-1 has been implicated in several CNS disorders and insults (Arzberger et al., 1997; Goodrich et al., 2013; Harvey et al., 2011; Rothstein et al., 1995; Tanaka et al., 1997), with the particular focus of this thesis being its role in AD, where GLT-1 expression is reduced by as much as 50% (Woltjer et al., 2010). Furthermore, synaptic loss has been identified as the strongest pathological correlate of cognitive decline in AD with evidence

that glutamatergic terminals are one of the most susceptible (Canas et al., 2014; DeKosky & Scheff, 1990; Kashani et al., 2008; Terry et al., 1991). Hence, glutamatergic dyshomeostasis is believed to play a role in the cognitive decline in AD and loss of GLT-1 function may be a primary mechanism of this dyshomeostasis.

Recent work from the Cook laboratory examined the effects of partial GLT-1 loss (GLT-1 heterozygosity) in mice harboring familial AD mutations in the amyloid precursor protein (APPswe) and Presenilin 1 (PS1ΔE9), where we found that loss of GLT-1 induced early-occurring cognitive deficits (Mookherjee et al., 2011). The previous chapter detailed how these deficits were associated with early changes to components of the insulin signaling cascade in the brain, an important regulatory pathway of cognitive and metabolic processes, before significant amyloid pathology presented (Meeker et al., 2015; Mookherjee et al., 2011). Furthermore, the CNS insulin signaling disturbances caused by partial GLT-1 loss in our mice are consistent with insulin signaling changes identified in human AD cases. For example, several studies have identified increased Akt phosphorylation and activity, decreased insulin receptor (IR) tyrosine phosphorylation, and increased insulin receptor substrate-1 (IRS-1) serine phosphorylation, which are believed to be indicative of brain insulin resistance (Griffin et al., 2005; Moloney et al., 2010; Rickle et al., 2004; Talbot et al., 2012).

Similar to findings of GLT-1 loss, insulin signaling changes develop early in the course of AD with reduced IR tyrosine phosphorylation and increased IRS-1 serine phosphorylation in particular being identified in individuals with mild cognitive impairment (MCI) (Talbot et al., 2012). Furthermore, basal activation states of insulin signaling molecules have been found to be highly related to cognitive ability in AD (Talbot et al., 2012). These results provide further evidence that these two pathologies, GLT-1 loss and insulin signaling disturbances, both occur

early and may interact in AD pathogenesis. However, the mechanistic link between GLT-1 dysfunction and alterations to components of the insulin signaling cascade in the brain is unknown.

Given that alterations to insulin signaling molecules in AD have been predominantly identified in neurons and the relatively equivalent extent of GLT-1-mediated uptake between astrocytes and neurons, we utilized primary cortical neurons to examine the effects of GLT-1 loss on insulin signaling. In this study, we found that inhibition of GLT-1 (and inhibition of both neuronal glutamate transporters, GLT-1 and EAAC1) led to significant reductions in the activation profiles of several proteins involved in insulin signaling under both basal and insulin-evoked conditions. Furthermore, we found that these changes could be reversed by both scavenging of extracellular glutamate and blockade of NMDA receptors, suggesting that regulation of endogenously released glutamate by neuronal GLT-1 is critical for maintenance of insulin signaling.

Methods

Primary Neuron Culture: Primary cortical neurons were prepared from postnatal day zero C57BL/6J pups. Briefly, pups were decapitated and cortices were dissected in Neurobasal®-A medium supplemented with 5 mM HEPES and 30 mM glucose. After dissection, cortices were trypsinized for 25 min. at 37°C and dissociated. Neurons were plated in Neurobasal®-A with B-27® supplement at a density of 1 x 10^6 cells/well in poly-D-lysine coated 6-well plates (used for Western blot analysis and glutathione assay). After 24 hours, neurons were changed to fresh complete media (Neurobasal®-A with B-27® supplement) with the addition of 8 μM 5-fluoro-2'-deoxyuridine (FUDR) and 20 μM uridine to inhibit glial cell

growth. All experiments involving animals were performed in accordance with procedures approved by the VAPSHCS Institutional Animal Care and Use Committee.

Drug Treatments and Lysate Preparation: After 15 days *in vitro* (DIV), cells were changed to Neurobasal®-A media lacking B-27® supplement and treated with the non-transportable GLT-1 specific inhibitor dihydrokainic acid (DHK) or the non-transportable pan-specific glutamate transporter inhibitor threo-β-benzyloxyaspartic acid (TBOA) for 5 hours under basal (no insulin treatment) or insulin-evoked conditions (100 nM insulin for the final 10 min. of treatments). For their respective experiments, the following drugs were applied to the neurons immediately prior to addition of DHK or TBOA and co-incubated for 5 hours: the glutamate scavenger glutamic pyruvic transaminase (GPT; 5 U/ml added with 5 mM pyruvate), L-alanine and α-ketoglutarate (0.5 mM each), the non-competitive NMDA receptor antagonist MK-801 (1 μM), the non-competitive low-affinity NMDA receptor antagonist memantine (20 μM), the GluN2B-specific NMDA receptor antagonist ifenprodil (10 μM), the broad-spectrum protein kinase C (PKC) inhibitor Gö 6983 (0.5 μM), the protein phosphatase 1/2A (PP1/PP2A)-specific inhibitor okadaic acid (4 nM), or the protein phosphatase 2B (PP2B) inhibitor FK 506 (100 nM). Insulin, GPT, pyruvate, L-alanine, and α-ketoglutarate were obtained from Sigma-Aldrich (St. Louis, MO) and all other drugs were obtained from Tocris (Bristol, United Kingdom).

After drug treatments, primary cortical neurons were harvested in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4) containing 1% Triton X-100, protease inhibitor cocktail, and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St. Louis, MO) and then collected by scraping. Cell lysates were then spun at 15,000 rpm for 30 min. at 4°C and the supernatant was used as the whole cell lysate. Lysates were stored at -70°C. Lysate protein

concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Western Blot Analysis: Western blot analysis was performed similarly as in the previous chapter with minor differences. Briefly, protein lysates were solubilized in Laemmli sample buffer, loaded onto 4-20% SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), electrophoresed, and transferred onto polyvinyl difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked in 5% (w/v) non-fat dried milk dissolved in Tris-buffered saline (20 mM Tris, 140 mM NaCl, pH 7.4) with 0.03% Tween-20 and subsequently incubated with specific primary antibodies against phospho-insulin receptor β-subunit (pIRβ, $Tyr^{1150/1151}$), total insulin receptor β-subunit (IRβ), phospho-Akt (pAkt, Ser^{473}), total Akt, phospho-IRS-1 (pIRS-1, detects $Ser^{632/635}$ in mice and $Ser^{636/639}$ in humans), total IRS-1, phospho-GSK-3β (pGSK-3β, Ser^{9}), total GSK-3β, phospho-mTOR (p-mTOR, Ser^{2448}), total mTOR, or pyruvate kinase (loading control) overnight at 4°C. All primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) with the exceptions of the pan-specific antibody to IRβ which was obtained from Millipore (Billerica, MA), and the anti-pyruvate kinase antibody that was obtained from Rockland Immunochemicals (Gilbertsville, PA). Western blots were then incubated in the corresponding horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and were developed using Amersham ECL Prime (GE Healthcare, Piscataway, NJ) and an ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ).

Densitometry analyses were performed using ImageQuant TL software (GE Healthcare, Piscataway, NJ). Band intensities of phosphorylated proteins were normalized to band intensities of corresponding total protein levels. Band intensities of total IRS-1 levels were also normalized

to band intensities of the loading control pyruvate kinase. For each protein examined (Akt, IRβ, IRS-1, GSK-3β, and mTOR) the ratios of phospho-epitope / total protein or total protein / pyruvate kinase were normalized to the control group as 100%.

Measurement of Glutathione: Total intracellular levels of glutathione (reduced GSH and oxidized GSSG) in primary cortical neuron lysates were determined using a Glutathione Assay Kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Lysates were centrifuged at 15,000 rpm for 30 min. at 4°C and the supernatant was used for the assay. Lysate protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Total glutathione levels in samples were estimated by measuring the absorbance at 405 nm using a μQuant microplate spectrophotometer (BioTek, Winooski, VT). Pure GSSG included in the kit was used to obtain a standard curve.

Statistics: Data are reported as mean ± standard error of the mean (SEM). Statistical significance between groups was assessed using a repeated-measures analysis of variance (ANOVA) on data before normalization to control values as 100%. Following a statistically significant repeated-measures ANOVA result ($p \leq 0.05$), Dunnett's multiple comparisons test was used to identify significant differences between each treatment group and the control group. Statistical analyses were carried out using SPSS (IBM, Armonk, NY) and Prism (GraphPad, La Jolla, CA) software.

Results

Inhibition of neuronal GLT-1 attenuates both the insulin-evoked and basal activation profiles of components of the insulin signaling pathway:

To determine the effects of inhibition of neuronal GLT-1 on insulin signaling, 15 days *in vitro* (DIV) primary cortical neurons were changed to media lacking B27 and were treated for 5 hours with the GLT-1 specific inhibitor DHK (500 μM) or the broad-spectrum glutamate transporter inhibitor TBOA (100 μM; to target both GLT-1 and the other neuronal glutamate transporter EAAC1). After 5 hours, neurons were either immediately lysed (basal signaling) or treated with 100 nM insulin for 10 minutes (insulin-evoked signaling). Lysates of cortical neurons were then subjected to Western blotting to determine phosphorylated and total levels of specific insulin signaling proteins. Insulin receptor activation was first determined by measuring the extent of insulin receptor β-subunit (IRβ) phosphorylation at $Tyr^{1150/1151}$, with increased phosphorylation of this site indicative of insulin receptor activation (White et al., 1988).

Under basal signaling conditions, we found that there were no significant effects of treatment with DHK or TBOA on the ratio of phospho-IRβ ($Tyr^{1150/1151}$) to total IRβ (Figure 3.1A and 3.1C; pIRβ/IRβ; $F[2,12]= 1.256$, n.s.). However, under insulin-evoked conditions, there was a significant difference in pIRβ/IRβ ($Tyr^{1150/1151}$) between groups (Figure 3.1B and 3.1C; $F[2,10]= 12.158$, $p= 0.002$). Post-hoc analysis using Dunnett's multiple comparisons test revealed that treatment with DHK caused a significant ($p= 0.029$) decrease in pIRβ/IRβ ($Tyr^{1150/1151}$) and treatment with TBOA led to a similar, but slightly greater decrease ($p< 0.001$). This suggests that inhibition of glutamate transport blunted the ability of insulin to activate its receptor. However, it is important to note that IRβ phosphorylation was only minimally detectable under basal conditions as B27 (which contains insulin) was not present in the treatment media, so any differences may be difficult to detect.

Downstream of the insulin receptor, several proteins are involved in carrying out the variety of cellular responses to insulin signaling, with the serine/threonine kinase Akt (also

known as protein kinase B) being a central node of signaling. Akt phosphorylation was measured by Western blot at Ser^{473}, with phosphorylation of this site being indicative of Akt activation. From this, we found a significant difference between groups in the ratio of phospho-Akt (Ser^{473}) to total Akt (pAkt/Akt) under both basal (Figure 3.2A and 3.2K; $F[2,12]= 64.517$, $p< 0.001$) and insulin-evoked (Figure 3.2D and 3.2K; $F[2,12]= 31.124$, $p< 0.001$) signaling conditions. Post-hoc analysis revealed that both DHK and TBOA led to significant reductions in basal (DHK: $p< 0.001$; TBOA: $p< 0.001$) and insulin-evoked (DHK: $p< 0.001$; TBOA: $p< 0.001$) ratios of pAkt/Akt.

We also examined the phosphorylation states of proteins regulated by Akt, including glycogen synthase kinase-3β (GSK-3β) and mammalian target of rapamycin (mTOR). Phosphorylation of GSK-3β at Ser^{9} reduces its activity whereas phosphorylation of mTOR at Ser^{2448} is indicative of its activation. Consistent with Akt being an important regulator of both GSK-3β and mTOR, we found that treatment with DHK or TBOA led to significant differences in the ratios of phospho-GSK-3β (Ser^{9}) to total GSK-3β (pGSK-3β/GSK-3β) and phospho-mTOR (Ser^{2448}) to total mTOR (p-mTOR/mTOR) under both basal (Figure 3.2B, 3.2C, and 3.2K; pGSK-3β/GSK-3β: $F[2,12]= 36.940$, $p< 0.001$; p-mTOR/mTOR: $F[2,12]= 46.301$, $p< 0.001$) and insulin-evoked (Figure 3.2E, 3.2F, and 3.2K; pGSK-3β/GSK-3β: $F[2,12]= 35.626$, $p< 0.001$; p-mTOR/mTOR: $F[2,12]= 15.011$, $p= 0.001$) conditions. Post-hoc analyses revealed that both DHK and TBOA caused significant reductions in pGSK-3β/GSK-3β under basal (DHK: $p< 0.001$; TBOA: $p< 0.001$) and insulin-evoked conditions (DHK: $p< 0.001$; TBOA: $p< 0.001$). Similarly, both DHK and TBOA caused significant reductions in p-mTOR/mTOR under basal (DHK: $p< 0.001$; TBOA: $p< 0.001$) and insulin-evoked conditions (DHK: $p= 0.048$; TBOA: $p< 0.001$).

The insulin receptor substrate-1 (IRS-1) is a critical adapter protein responsible for transmission of signals from the insulin receptor to downstream proteins, such as Akt (Bassil et al., 2014; Kleinridders et al., 2014). Serine phosphorylation of IRS-1 is a mechanism of inactivation of insulin signaling by promoting dissociation of IRS-1 from the insulin receptor, and can lead to IRS-1 degradation (Boura-Halfon & Zick, 2009; Hotamisligil et al., 1996). However, we did not find a significant difference in the ratio of phospho-IRS-1 ($Ser^{632/635}$) to total IRS-1 (pIRS-1/IRS-1) under either basal (Figure 3.2G and 3.2K; $F[2,10]= 3.112$, n.s.) or insulin-evoked conditions (Figure 3.2I and 3.2K; $F[2,10]= 0.489$, n.s.). However, total IRS-1 levels normalized to the loading control pyruvate kinase (PK) were found to be significantly different between groups under both basal (Figure 3.2H and 3.2K; $F[2,12]= 26.855$, $p< 0.001$) and insulin-evoked conditions (Figure 3.2J and 3.2K; $F[2,12]= 21.472$, $p< 0.001$). Post-analysis determined that both DHK and TBOA treatment significantly reduced total IRS-1 levels under basal (DHK: $p= 0.014$; TBOA: $p< 0.001$) and insulin-evoked conditions (DHK: $p= 0.009$; TBOA: $p< 0.001$). This suggests that inhibition of glutamate transport may attenuate IRS-1 signaling by promoting its degradation, potentially preventing the ability to detect changes in serine phosphorylation.

Scavenging of extracellular glutamate prevents insulin signaling changes induced by inhibition of GLT-1:

Glutamate transporters play a critical role in rapidly removing glutamate after release, making it probable that inhibition of GLT-1 leads to accumulation of extracellular glutamate. We wanted to determine if scavenging of extracellular glutamate by the enzyme glutamic-pyruvic transaminase (GPT) would prevent the insulin signaling changes induced by inhibition of GLT-1. GPT catalyzes the conversion of L-glutamate and pyruvate to L-alanine and α-ketoglutarate.

Consequently, to scavenge extracellular glutamate during DHK and TBOA treatments, GPT (5 U/ml) was added with an excess of pyruvate (5 mM). Since similar insulin signaling alterations occurred under both basal and insulin-evoked conditions, we examined phosphorylation states of insulin signaling proteins under basal conditions for the remainder of experiments.

We found statistically significant differences between groups (Figure 3.3) in the ratios of pAkt/Akt (Figure 3.3A and 3.3F; $F[4,12]= 19.900$, $p< 0.001$), pGSK-3β/GSK-3β (Figure 3.3B and 3.3F; $F[4,12]= 66.155$, $p< 0.001$), p-mTOR/mTOR (Figure 3.3C and 3.3F; $F[4,12]= 5.292$, $p= 0.011$), and total IRS-1 levels (Figure 3.3E and 3.3F; $F[4,12]= 15.291$, $p< 0.001$) . As expected, post-hoc analysis revealed that treatments with DHK or TBOA led to significant reductions in the levels of pAkt/Akt (DHK: $p= 0.003$; TBOA: $p< 0.001$), pGSK-3β/GSK-3β (DHK: $p= 0.009$; TBOA: $p< 0.001$), and total IRS-1 levels (DHK: $p= 0.023$; TBOA: $p< 0.001$). However, the levels of pAkt/Akt, pGSK-3β/GSK-3β, or total IRS-1 were not significantly different from control when DHK and TBOA were each co-incubated with GPT (and pyruvate). While both DHK and TBOA both caused similar reductions in the levels of p-mTOR/mTOR as seen previously, only the effect of TBOA was significant ($p= 0.008$). Furthermore, the levels of p-mTOR/mTOR were not significantly different between control and DHK/TBOA groups that were co-incubated with GPT. There was no significant difference between groups in the levels of pIRS-1/IRS-1 (Figure 3.3D and 3.3F; $F[4,8]= 1.700$, n.s.).

To confirm that the effect of the presence of GPT is due to scavenging of glutamate and not production of alanine and α-ketoglutarate, we applied alanine (0.5 mM) and α-ketoglutarate (0.5 mM) together during DHK/TBOA treatments (Figure 3.4). We found statistically significant differences between groups in the ratios of pAkt/Akt (Figure 3.4A and 3.4F; $F[4,8]= 8.140$, $p= 0.006$), pGSK-3β/GSK-3β (Figure 3.4B and 3.4F; $F[4,8]= 8.490$, $p= 0.006$), p-mTOR/mTOR

(Figure 3.4C and 3.4F; F[4,8]= 6.832, p= 0.011), total IRS-1 (Figure 3.4E and 3.4F; F[4,8]= 49.487, p< 0.001), and pIRS-1/IRS-1(Figure 3.4D and 3.4F; F[4,4]= 7.531, p= 0.038). Unlike GPT treatment, co-incubation of DHK and TBOA with L-alanine/α-ketoglutarate resulted in similar reductions as DHK or TBOA treatment alone relative to control in the levels of pAkt/Akt (DHK: p= 0.049; DHK + L-alanine/α-ketoglutarate: p= 0.006; TBOA: p= 0.007; TBOA + L-alanine/α-ketoglutarate: p= 0.004), pGSK-3β/GSK-3β (DHK: n.s.; DHK + L-alanine/α-ketoglutarate: p= 0.025; TBOA: p= 0.005; TBOA + L-alanine/α-ketoglutarate: p= 0.003), p-mTOR/mTOR (DHK: n.s.; DHK + L-alanine/α-ketoglutarate: p= 0.016; TBOA: p= 0.008; TBOA + L-alanine/α-ketoglutarate: p= 0.009), and total IRS-1 (DHK: p= 0.002; DHK + L-alanine/α-ketoglutarate: p< 0.001; TBOA: p< 0.001; TBOA + L-alanine/α-ketoglutarate: p< 0.001). It should be noted, however, that DHK on its own trended toward, but did not result in significant reductions in the levels of pGSK-3β/GSK-3β or p-mTOR/mTOR, which is not surprising given that the levels of pGSK-3β/GSK-3β and p-mTOR/mTOR change much less dramatically than those of pAkt/Akt or total IRS-1. Furthermore, while there was a significant difference between groups in the levels of pIRS-1/IRS-1, the changes were modest and only TBOA co-incubated with L-alanine/α-ketoglutarate was found to be significantly different from control (p= 0.039).

NMDA receptor inhibition prevents insulin signaling changes induced by inhibition of GLT-1:

The findings above suggest that inhibition of GLT-1 prevents glutamate uptake thereby causing an accumulation of extracellular glutamate, which leads to the insulin signaling changes observed. However, the site of action of the glutamate is unknown. Previous studies have shown that Akt phosphorylation can be modulated by N-methyl-D-aspartate (NMDA)-type glutamate receptors (Shehata et al., 2012; Sutton & Chandler, 2002; Y. Wang et al., 2014). To determine if

NMDA receptor activation is involved with insulin signaling changes induced by GLT-1 inhibition, we applied the uncompetitive NMDA receptor antagonist MK-801 (1 μM) in conjunction with DHK/TBOA treatments (Figure 3.5). We found significant differences between groups in the ratios of pAkt/Akt (Figure 3.5A and 3.5F; $F[4,8]= 11.141$, $p= 0.002$), pGSK-3β/GSK-3β (Figure 3.5B and 3.5F; $F[4,8]= 4.073$, $p= 0.043$), p-mTOR/mTOR (Figure 3.5C and 3.5F; $F[4,8]= 7.286$, $p= 0.009$), and total IRS-1/PK (Figure 3.5E and 3.5F; $F[4,8]= 16.595$, $p= 0.001$). Surprisingly, neither DHK nor TBOA resulted in statistically significant reductions in the levels of pAkt/Akt or pGSK-3β/GSK-3β by post-hoc analysis. However, similar reductions in pAkt/Akt and pGSK-3β/GSK-3β by DHK and TBOA can visibly identified whereas co-incubation with MK-801 resulted in pAkt/Akt and pGSK-3β/GSK-3β levels comparable to and not statistically different than those of the control. However, both DHK and TBOA did cause significant reductions in p-mTOR/mTOR (DHK: $p= 0.024$; TBOA: $p= 0.003$) and total IRS-1 levels (DHK: $p= 0.035$; TBOA: $p= 0.015$), while p-mTOR/mTOR and total IRS-1 levels were not significantly different from control when DHK and TBOA were co-incubated with MK-801. Furthermore, the levels of pIRS-1/IRS-1 were not significantly different between groups (Figure 3D and 3F; $F[4,8]= 2.960$, n.s.).

To further test the hypothesis that inhibition of GLT-1 results in NMDA receptor activation and disturbances to insulin signaling, we also tested the low-affinity, voltage-dependent uncompetitive NMDA receptor antagonist memantine (20 μM), which is currently approved for the treatment of AD (Figure 3.6). From this, we found significant differences between groups in the ratios of pAkt/Akt (Figure 3.6A and 3.6F; $F[4,12]= 11.396$, $p< 0.001$), pGSK-3β/GSK-3β (Figure 3.6B and 3.6F; $F[4,12]= 4.866$, $p= 0.015$), p-mTOR/mTOR (Figure 3.6C and 3.6F; $F[4,12]= 3.340$, $p= 0.047$), and total IRS-1/PK (Figure 3.6E and 3.6F; $F[4,12]=$

3.651, p= 0.036). Through post-hoc analysis, we found that DHK and TBOA caused significant reductions in the ratio of pAkt/Akt (DHK: p= 0.012; TBOA: p= 0.001) while only TBOA caused significant reductions in the levels of pGSK-3β/GSK-3β (p= 0.011), p-mTOR/mTOR (p= 0.04), and total IRS-1 (p= 0.036). However, when co-incubated with memantine, neither DHK nor TBOA treatment resulted in significant reductions to pAkt/Akt, pGSK-3β/GSK-3β, p-mTOR/mTOR, or total IRS-1 as compared to control. Furthermore, pIRS-1/IRS-1 levels were modestly, but significantly different between groups (Figure 3.6D and 3.6F; F[4,12]= 6.012, p= 0.007), although post-hoc analysis did not find any individual group significantly different from control.

Preferential inhibition of GluN2B-containing NMDA receptors prevents insulin signaling changes induced by inhibition of GLT-1:

In recent years, interest has been growing in the potential role of extrasynaptic NMDA receptors in AD as they have been found to couple to pathways involved in promoting cell death and dysfunction (Hardingham & Bading, 2010; Parsons & Raymond, 2014; X. Zhou et al., 2013). In our system, it is feasible that extrasynaptic NMDA receptors play a role in the effects induced by GLT-1 inhibition as glutamate is likely able to diffuse to extrasynaptic sites since clearance is reduced. Consistent with this, others have suggested that inhibition of glutamate uptake preferentially targets activation of GluN2B-containing receptors (Jarzylo & Man, 2012; Nie & Weng, 2009) and there is evidence that GluN2B-containing receptors are preferentially localized to extrasynaptic sites (Groc et al., 2006; J. H. Li et al., 1998; Tovar & Westbrook, 1999). Thus, we wanted to determine if the insulin signaling changes induced by GLT-1 inhibition could be reversed by specific inhibition of GluN2B-containing NMDA receptors.

To accomplish this, we utilized the GluN2B-specific NMDA receptor antagonist ifenprodil (10 μM; Figure 3.7). Ratios of pAkt/Akt (Figure 3.7A and 3.7F; $F[4,16]= 8.648$, $p< 0.001$), pGSK-3β/GSK-3β (Figure 3.7B and 3.7F; $F[4,16]= 3.923$, $p= 0.021$), p-mTOR/mTOR (Figure 3.7C and 3.7F; $F[4,12]= 9.451$, $p= 0.001$), and total IRS-1 (Figure 3.7E and 3.7F; $F[4,16]= 10.322$, $p< 0.001$) were found to be significantly different between groups. Post-hoc analysis showed that both DHK and TBOA caused significant reductions in pAkt/Akt (DHK: $p= 0.009$; TBOA: $p< 0.001$), p-mTOR/mTOR (DHK: $p= 0.03$; TBOA: $p< 0.001$), and total IRS-1 (DHK: $p= 0.021$; TBOA: $p< 0.001$) compared to control while only TBOA caused a significant reduction in pGSK-3β/GSK-3β ($p= 0.01$). Similar to both MK-801 and memantine, when co-incubated with ifenprodil, neither DHK nor TBOA resulted in significant reductions to pAkt/Akt, pGSK-3β/GSK-3β, p-mTOR/mTOR, or total IRS-1 levels. Moreover, pIRS-1/IRS-1 levels were not significantly different between groups (Figure 3.7D and 3.7F; $F[4,12]= 1.085$, n.s.).

Protein kinase C is not responsible for insulin signaling changes induced by inhibition of GLT-1:

NMDA receptor activation can activate several intracellular targets, one of which being protein kinase C (PKC) (Brennan-Minnella et al., 2013; Hori et al., 2005). Interestingly, other studies have shown that PKC can regulate both GLT-1 and EAAC1 expression (Gonzalez et al., 2002; Kalandadze et al., 2002). Furthermore, several isoforms of PKC have been shown to negatively regulate insulin signaling (De Fea & Roth, 1997; Y. Li et al., 2004; Y. F. Liu et al., 2001). Such data prompted us to address whether NMDA receptor activation is linked to changes in insulin signaling through activation of PKC. To test this hypothesis, we applied the broad-spectrum PKC inhibitor Gö 6983 (0.5 μM) during DHK/TBOA treatments (Figure 3.8).

We found significant differences between groups in the levels of pAkt/Akt (Figure 3.8A and 3.8F; $F[4,8]= 59.905$, $p< 0.001$), pGSK-3β/GSK-3β (Figure 3.8B and 3.8F; $F[4,8]= 36.239$,

$p < 0.001$), p-mTOR/mTOR (Figure 3.8C and 3.8F; $F[4,8] = 3.688$, $p = 0.05$), and total IRS-1 (Figure 3.8E and 3.8F; $F[4,8] = 10.693$, $p = 0.003$). However, unlike with NMDA receptor antagonists, post-hoc analysis revealed that co-incubation of Gö 6983 with DHK and TBOA resulted in similar reductions as DHK or TBOA treatment alone in the levels of pAkt/Akt (DHK: $p < 0.001$; DHK + Gö 6983: $p < 0.001$; TBOA: $p < 0.001$; TBOA + Gö 6983: $p < 0.001$), pGSK-3β/GSK-3β (DHK: $p < 0.001$; DHK + Gö 6983: $p < 0.001$; TBOA: $p < 0.001$; TBOA + Gö 6983: $p < 0.001$), p-mTOR/mTOR (DHK: n.s.; DHK + Gö 6983: n.s.; TBOA: $p = 0.037$; TBOA + Gö 6983: $p = 0.034$), and total IRS-1 (DHK: $p = 0.05$; DHK + Gö 6983: $p = 0.029$; TBOA: $p = 0.002$; TBOA + Gö 6983: $p = 0.002$). Levels of pIRS-1/IRS-1 were not significantly different between groups (Figure 3.8D and 3.8F; $F[4,8] = 2.494$, n.s.).

Inhibition of protein phosphatases PP1/PP2A restores insulin signaling, but in a different pattern than NMDA receptor inhibition:

Activation of NMDA receptors can induce activation of several different protein phosphatases. Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) in particular have been implicated in regulation of Akt phosphorylation after NMDA receptor activation (Nakazawa et al., 2005; Shehata et al., 2012). To determine if PP1/PP2A were involved in regulation of insulin signaling after GLT-1 inhibition, we applied the PP1/PP2A antagonist okadaic acid (4 nM) during DHK/TBOA treatments (Figure 3.9). The levels of pAkt/Akt were found to be significantly different between groups (Figure 3.9A and 3.9F; $F[4,8] = 12.005$, $p = 0.002$). This difference was due to significant reductions induced by DHK ($p = 0.024$) and TBOA ($p = 0.002$) treatment, while DHK and TBOA treatments co-incubated with okadaic acid were not significantly different from control. This result is similar to the pattern seen when scavenging extracellular glutamate or application of MK-801, memantine, and ifenprodil.

However, okadaic acid dramatically altered the effects of DHK and TBOA treatment on pGSK-3β/GSK-3β and p-mTOR/mTOR levels resulting in patterns dissimilar to the other inhibitors. For example, there were significant differences between groups in in pGSK-3β/GSK-3β (Figure 3.9B and 3.9F; $F[4,8]= 26.106$, $p< 0.001$) and p-mTOR/mTOR (Figure 3.9C and 3.9F; $F[4,8]= 7.577$, $p= 0.008$) compared to control. However, post-hoc analysis revealed that only okadaic acid significantly increased the levels of pGSK-3β/GSK-3β (DHK + okadaic acid: $p< 0.001$; TBOA + okadaic acid: $p= 0.002$) while neither DHK nor TBOA alone had significant effects. Furthermore, none of the individual group levels of p-mTOR/mTOR were found to be significantly different from control. While okadaic acid appeared to prevent reductions in total IRS-1 levels it also seemed to increase pIRS-1/IRS-1 above that of DHK or TBOA alone, although there were no overall differences between groups for either total IRS-1 (Figure 3.9E and 3.9F; $F[4,8]= 2.073$, n.s.) or pIRS/IRS-1 (Figure 3.9D and 3.9F; $F[4,8]= 2.322$, n.s.).

To determine if other protein phosphatases may be involved in the insulin signaling changes induced by inhibition of GLT-1, we also applied the protein phosphatase 2B (PP2B; also known as calcineurin) antagonist FK 506 (100 nM) during DHK/TBOA treatments (Figure 3.10). From this, we found significant differences between groups in pAkt/Akt (Figure 3.10A and 3.10F; $F[4,8]= 45.839$, $p< 0.001$), pGSK-3β/GSK-3β (Figure 3.10B and 3.10F; $F[4,8]= 4.016$, $p= 0.045$), p-mTOR/mTOR (Figure 3.10C and 3.10F; $F[4,8]= 5.931$, $p= 0.016$), and total IRS-1 levels (Figure 3.10E and 3.10F; $F[4,8]= 34.994$, $p< 0.001$). However, unlike okadaic acid but similar to Gö 6983 treatment, DHK and TBOA had similar effects on pAkt/Akt (DHK: $p< 0.001$; DHK + FK 506: $p< 0.001$; TBOA: $p< 0.001$; TBOA + FK 506: $p< 0.001$), pGSK-3β/GSK-3β (DHK: n.s.; DHK + FK 506: n.s.; TBOA: $p= 0.033$; TBOA + FK 506: $p= 0.023$), p-mTOR/mTOR (DHK: $p= 0.037$; DHK + FK 506: $p= 0.029$; TBOA: $p= 0.01$; TBOA + FK 506:

p= 0.011), and total IRS-1 (DHK: p< 0.001; DHK + FK 506: p= 0.001; TBOA: p< 0.001; TBOA + FK 506: p< 0.001) whether or not they were co-incubated with FK 506. Furthermore, there were no significant differences in pIRS-1/IRS-1 between groups (Figure 3.10D and 3.10F; F[4,8]= 0.847, n.s.).

Inhibition of both GLT-1 and EAAC1, but not GLT-1 alone, reduces total glutathione levels:

Along with their critical role in regulating extracellular glutamate levels, glutamate transporters are required for uptake of cysteine in mature neurons, the rate-limiting factor in the synthesis of the antioxidant glutathione (Aoyama et al., 2006; Chen & Swanson, 2003). Thus, inhibition of GLT-1 may interfere with glutathione levels leading to alterations in components of the insulin signaling pathway due to changes in the oxidative state of the neurons. While we found a statistically significant difference between groups in the levels of total glutathione (Figure 3.11; F[2,4]= 22.78, p= 0.007), only TBOA treatment significantly reduced total glutathione (p= 0.006) while DHK treatment had no significant effect compared to control. Thus, we found that inhibition of GLT-1 alone did not alter total glutathione levels whereas inhibition of both GLT-1 and EAAC1 led to a significant reduction in total glutathione.

Discussion

Uptake of endogenously released glutamate by transporters is the principal mechanism for controlling its extracellular concentration. Transporters are not only essential to prevent overt glutamate excitotoxicity but are also critical for regulating the spatial and temporal profile of glutamatergic signaling. Not surprisingly, loss and dysfunction of glutamate transporters, particularly GLT-1, have been implicated in several CNS disorders and may contribute to neuronal and synaptic dysfunction and/or loss. Given their role in modulating glutamatergic signaling, we have hypothesized that loss or dysfunction of glutamate transporters results in

alterations in cellular signaling, which may play a role in neuronal dysfunction. In keeping with this, we have previously shown that in an animal model of AD, partial loss of GLT-1 accelerated the onset of cognitive deficits before the onset of significant amyloid pathology (Mookherjee et al., 2011). These changes in cognition were accompanied by early-occurring disturbances in insulin signaling in the brain, a pathway important for regulation of both metabolic and cognitive processes (Meeker et al., 2015). However, using an *in vivo* model we were unable to determine if insulin signaling alterations induced by partial GLT-1 loss were directly caused by dysregulation of glutamatergic signaling or where these changes were occurring.

GLT-1 is expressed predominantly in astrocytes in the CNS, yet insulin signaling changes identified in AD patients have been primarily attributed to neurons. This raises the possibility that partial GLT-1 loss from astrocytes results in insulin signaling disturbances manifested by neurons. However, it has recently been established that GLT-1 is also expressed in neurons, albeit at markedly lower levels than astrocytes. Intriguingly, recent work has shown that GLT-1 expressed in neuronal synaptic terminals accounts for an equivalent amount of uptake as astrocytes even though it represents only 10% or less of total GLT-1 protein in the brain (Furness et al., 2008). Furthermore, neuronal GLT-1 has been found to be responsible for a much greater proportion of synaptosomal glutamate uptake than astrocytic GLT-1 (Petr et al., 2015). Consequently, it is also possible that partial loss of neuronal GLT-1 may have resulted in neuronal insulin signaling disturbances. In this chapter, we utilized primary cortical neuron cultures to determine if loss of neuronal GLT-1 function results in similar insulin signaling alterations in neurons as those seen *in vivo* and how this can mechanistically occur.

Inhibition of neuronal GLT-1 produces insulin signaling alterations similar to those seen in Alzheimer's disease

Several studies have identified insulin signaling disturbances in brains of AD patients, which have been discussed more thoroughly in other parts of this thesis. In the cortex and hippocampus, AD patients show reduced insulin receptor tyrosine phosphorylation and increased serine phosphorylation of IRS-1 under basal signaling conditions (Moloney et al., 2010; Talbot et al., 2012). Furthermore, using *ex vivo* hippocampal slices, one study also found a reduced ability of insulin to stimulate the insulin receptor and IRS-1 in AD cases compared to normal controls (Talbot et al., 2012). Together these findings are indicative of reduced insulin signaling in these brain regions in AD and are interpreted as a form of brain insulin resistance. Consistent with insulin signaling changes identified in human AD cases, inhibition of neuronal GLT-1 resulted in blunted insulin-evoked IRβ phosphorylation ($Tyr^{1150/1151}$). Moreover, while serine phosphorylation of IRS-1 ($Ser^{632/635}$ – equivalent to $Ser^{636/639}$ in humans) was not significantly altered, total IRS-1 levels were significantly reduced by GLT-1 inhibition. IRS-1 serine phosphorylation can lead to its degradation and a reduction in total IRS-1 levels in AD brains has been previously identified (Moloney et al., 2010).

Downstream of the insulin receptor, we also determined the phosphorylation states of several insulin signaling proteins that have been implicated in AD. Inhibition of neuronal GLT-1 *in vitro* led to a reduction in Akt (Ser^{473}) phosphorylation. In accordance with the role of Akt in regulating GSK-3β and mTOR, phosphorylation of GSK-3β (Ser^{9}) and mTOR (Ser^{2448}) were also found to be reduced by GLT-1 inhibition. Intriguingly, while the reductions in Akt, GSK-3β, and mTOR phosphorylation are consistent with the reduced insulin signaling identified at the level of IRβ and IRS-1, several studies have shown that basal phosphorylation levels and activity of Akt are elevated in AD brains and Akt (Ser^{473}) phosphorylation was increased by partial GLT-1 loss *in vivo* (Griffin et al., 2005; Meeker et al., 2015; Pei et al., 2003; Rickle et al., 2004; Talbot et al.,

2012). Furthermore, basal phosphorylation states of GSK-3β and mTOR have typically been found to be either unchanged or increased in AD (Griffin et al., 2005; X. Li et al., 2005; Pei et al., 1997; Steen et al., 2005; Talbot et al., 2012).

The discordance between decreased IRβ/IRS-1 signaling and increased downstream signaling through Akt, GSK-3β, and mTOR being found together in AD has yet to be fully resolved. However, several studies have shown that activation of Akt and mTOR can lead to negative feedback regulation of insulin signaling through serine phosphorylation of IRS-1 (Morisco et al., 2005; Ozes et al., 2001). This negative feedback loop may be best appreciated in a chronic, *in vivo* system where other factors (e.g. Aβ oligomers, interaction with other cell types, peripheral factors from vasculature) may be interacting with the insulin signaling pathway at the level of Akt, as it can be modulated by other pathways as well. This may be illustrated by the finding of elevated basal phosphorylation levels of Akt (Ser^{473}) and mTOR (Ser^{2448}) in AD hippocampal slices (GSK-3β-Ser^{9} levels were not significantly altered), however, when the hippocampal slices from AD cases were stimulated with insulin they showed blunted phosphorylation of Akt (Ser^{473}), GSK-3β (Ser^{9}), and mTOR (Ser^{2448}) compared to normal controls (Talbot et al., 2012). Furthermore, one study has shown that regulation of Akt phosphorylation by NMDA receptor activation is bi-directional and dependent on the level of synaptic activity (Sutton & Chandler, 2002), suggesting that glutamatergic regulation of Akt is complex and the direction of regulation may be dependent on several factors.

Collectively, these findings suggest that loss of neuronal GLT-1 (and similarly EAAC1 which will be discussed further later) function leads to a blunted ability of insulin to stimulate its cognate receptor and signal to downstream proteins, similar to changes identified in AD brains. Furthermore, this attenuation of insulin signaling by loss of GLT-1 function occurs through a

reduction in the basal phosphorylation profiles of insulin signaling proteins and degradation of IRS-1.

Loss of GLT-1 function results in glutamate dyshomeostasis and altered NMDA receptor activation

Given that the primary role of GLT-1 is to regulate extracellular glutamate concentration, we hypothesized that loss of GLT-1 function would lead to an accumulation of extracellular glutamate concentration and resulting alterations in glutamatergic and insulin signaling. Supporting this hypothesis, we found that scavenging of extracellular glutamate prevented the insulin signaling disturbances induced by inhibition of GLT-1. This is consistent with the findings by others that neuronal GLT-1 is responsible for a significant portion of glutamate uptake (Furness et al., 2008; Petr et al., 2015), and thus loss of GLT-1 function allows for a buildup of extracellular glutamate.

As other previous studies have suggested that activation of the NMDA receptor can regulate the phosphorylation levels of Akt and IRβ (Shehata et al., 2012; Sutton & Chandler, 2002; W. Q. Zhao et al., 2008), the NMDA receptor seemed to be the most likely target connecting elevated extracellular glutamate with insulin signaling changes. The involvement of the NMDA receptor in our effects was confirmed by our findings that both NMDA receptor antagonists MK-801 and memantine prevented insulin signaling disturbances induced by GLT-1 inhibition. Interestingly, memantine is one of the few therapeutics currently approved for the treatment of AD. Our current results suggest that memantine, at least partially, exerts its role as a therapeutic by preventing insulin signaling disturbances that are induced by loss of GLT-1 function and the resulting aberrant glutamatergic signaling.

As noted earlier, the NMDA receptor is a heterotetramer typically composed of two GluN1 (also denoted as NR1) subunits and two GluN2 (also denoted as NR2) subunits (J. H. Li et al., 1998). Several subunit isoforms exist that each gives the NMDA receptor unique distribution and functional characteristics, particularly the GluN2 isoforms (Hardingham & Bading, 2010; J. H. Li et al., 1998). For example, NMDA receptors containing the GluN2B subunit have found to be localized predominantly to extrasynaptic sites (Groc et al., 2006; Hardingham & Bading, 2010; J. H. Li et al., 1998; Tovar & Westbrook, 1999). These extrasynaptic receptors have also been found to couple to different signaling pathways than synaptic receptors (Hardingham & Bading, 2010; Parsons & Raymond, 2014). Consequently, when extracellular glutamate concentration is high enough or is not being managed properly and is able to diffuse out of the synapse and activate extrasynaptic receptors, this may result in different cellular responses from glutamate acting only at synaptic receptors. As glutamate transporters are critical for preventing the spread of glutamate out of the synapse or to neighboring synapses, we wanted to determine if activation of extrasynaptic NMDA receptors played a role in our effects. From this, we found that the GluN2B-selective NMDA receptor antagonist ifenprodil prevented the insulin signaling changes induced by GLT-1 inhibition, similar to MK-801 and memantine.

While these results suggest that inhibition of GLT-1 leads to activation of extrasynaptic NMDA receptors resulting in disturbances to insulin signaling, it should be noted that GluN2B-containing NMDA receptors show expression at synaptic sites as well and there is some debate as to whether GluN2A- and GluN2B-containing receptors actually localize to distinct sites (A. Z. Harris & Pettit, 2007; Petralia et al., 2010). However, other studies have shown that memantine also preferentially targets extrasynaptic receptors (Xia et al., 2010), giving further support to the

involvement of extrasynaptic NMDA receptors in GLT-1-mediated regulation of insulin signaling. The reversal of effects seen by inhibition of GluN2B-containing NMDA receptors may also be related to differential signaling properties, rather than changes in localization, conferred by the GluN2B subunits. For example, GluN2B-containing NMDA receptors have a higher affinity for glutamate and slower deactivation kinetics compared to GluN2A-type receptors, making GluN2B-type receptors better suited for tonic activation (Laurie & Seeburg, 1994; J. H. Li et al., 1998; Monyer et al., 1994). The insulin signaling changes induced by inhibition or dysfunction of GLT-1 may be due to tonic, rather than phasic, activation of NMDA receptors as insulin signaling changes were persistent across different time points (data not shown) and were the most apparent at longest time point examined (5 hours).

The link between NMDA receptor activation and insulin signaling alterations remains unresolved

Activation of the NMDA receptor is linked to variety of downstream signaling responses. In this *in vitro* setting, we attempted to identify the link between NMDA receptor activation and resulting inhibition of insulin signaling. PKC was an interesting target for several reasons, including its role in regulating GLT-1/EAAC1 expression (Gonzalez et al., 2002; Kalandadze et al., 2002), several isoforms of PKC have been found to negatively regulate insulin signaling (De Fea & Roth, 1997; Y. Li et al., 2004; Y. F. Liu et al., 2001), and that NMDA receptor signaling can lead to activation of PKC (Brennan-Minnella et al., 2013; Hori et al., 2005). However, even given these links with several components of our pathway, PKC inhibition did not alter the effects of GLT-1 inhibition on insulin signaling.

In other studies that have identified a relationship between NMDA receptor activation and Akt phosphorylation, it has been suggested that these changes were regulated by the protein

phosphatases PP1/PP2A (Nakazawa et al., 2005; Shehata et al., 2012). In keeping with these findings, inhibition of both PP1 and PP2A by okadaic acid prevented reductions in Akt phosphorylation induced by GLT-1 inhibition similar to NMDA receptor antagonists. However, at this same dose of okadaic acid, other components of the pathway were regulated much differently than Akt. For example, inhibition of PP1/PP2A caused robust increases in GSK-3β and mTOR phosphorylation and appeared to increase serine phosphorylation of IRS-1, suggesting that PP1/PP2A have a strong, tonic inhibitory role on insulin signaling proteins that may be independent of NMDA receptor activation. However, this role seemed specific to PP1/PP2A as inhibition of PP2B did not have effects on insulin signaling after GLT-1 inhibition.

Thus, while the protein phosphatases PP1/PP2A play a strong regulatory role on insulin signaling in neurons, it is likely either completely or at least partially independent of NMDA receptor activation. Furthermore, neither PKC nor PP2B activation are responsible for the decreases in insulin signaling induced by GLT-1 inhibition. Consequently, the exact link between NMDA receptor activation and inhibition of insulin signaling in our system still remains unknown.

Cooperative role of GLT-1 and EAAC1 in modulating insulin signaling through glutamate uptake

In all of the effects discussed above that were produced by inhibition of GLT-1 using DHK, treatment with TBOA produced similar and often greater effects. Given that our experiments were performed in purified primary neuron cultures, GLT-1 and EAAC1 are the predominant glutamate transporters present. This suggests that neuronal GLT-1 and EAAC1 are performing similar roles in modulating insulin signaling through regulation of glutamate. However, given that TBOA inhibits both GLT-1 and EAAC1, we cannot determine if loss of

EAAC1 alone results in similar disturbances to insulin signaling as GLT-1 loss or if the increased effects of TBOA are attributed to a synergistic effect given that all glutamate transport is shut down. Interestingly, GLT-1 and EAAC1 have been found to localize to different domains in neurons. GLT-1 is dispersed across relatively long distances on axons and in axon terminals while EAAC1 is localized to dendritic spines and the cell soma (Furness et al., 2008; Holmseth et al., 2012), potentially suggesting that each may be responsible for regulation of glutamate in their respective domains.

Regulation of oxidative stress in neurons by GLT-1 and EAAC1

In conjunction with regulating extracellular glutamate concentration, neuronal glutamate transporters in particular play a critical role in reducing oxidative stress through their ability to transport cysteine (Aoyama et al., 2006; Chen & Swanson, 2003). Cysteine is the rate-limiting substrate for the synthesis of the antioxidant glutathione (Chen & Swanson, 2003). Loss or reduction of neuronal glutathione could lead to increased oxidative stress and thus it was important to determine if insulin signaling disturbances induced by inhibition of neuronal GLT-1 were caused by depletion of total glutathione levels and resulting oxidative stress. However, inhibition of GLT-1 alone did not significantly alter total glutathione levels. Interestingly, inhibition of both neuronal transporters caused a significant drop in total glutathione, suggesting that either EAAC1 may play a more dominant role in neuronal cysteine uptake than GLT-1 as argued by Swanson and colleagues or that each individual transporter can maintain total glutathione levels on its own implying redundancy. Regardless, we conclude that alterations in insulin signaling induced by inhibition of neuronal GLT-1 are not caused by depletion of total glutathione. This provides further support that loss of neuronal GLT-1 function leads to insulin signaling disturbances directly through glutamate dyshomeostasis.

Conclusions:

Specific inhibition of neuronal GLT-1 alters the activation profiles of several key proteins involved with insulin signaling, including IRβ, IRS-1, Akt, GSK-3β, and mTOR. Furthermore, inhibition of both neuronal glutamate transporters, GLT-1 and EAAC1, led to similar alterations in insulin signaling proteins but to a slightly greater extent suggesting that both transporters are playing a similar, cooperative role. Co-treatment with the glutamate scavenger glutamic-pyruvic transaminase (GPT) prevented the changes induced by DHK/TBOA treatments, indicating that signaling changes induced by inhibition of neuronal GLT-1 (and EAAC1) are due to an accumulation of extracellular glutamate. This hypothesis was further supported by our finding that NMDA receptor blockade, likely those located at extrasynaptic sites, also prevented changes induced by neuronal glutamate transporter inhibition. Collectively, these findings provide some of the first evidence for a specific role of neuronally expressed GLT-1, which has now been identified to be responsible for a significant portion of autocrine/paracrine regulation of endogenously released glutamate. When unregulated, through loss of GLT-1 function, glutamate can activate NMDA receptors leading to a reduction in insulin signaling. Both GLT-1/EAAC1 dysfunction and insulin signaling deficiencies in the CNS have been implicated in neurological disorders, most prominently AD. These findings suggest that interactions between glutamate clearance and insulin signaling pathways in the CNS could have important implications for AD pathogenesis.

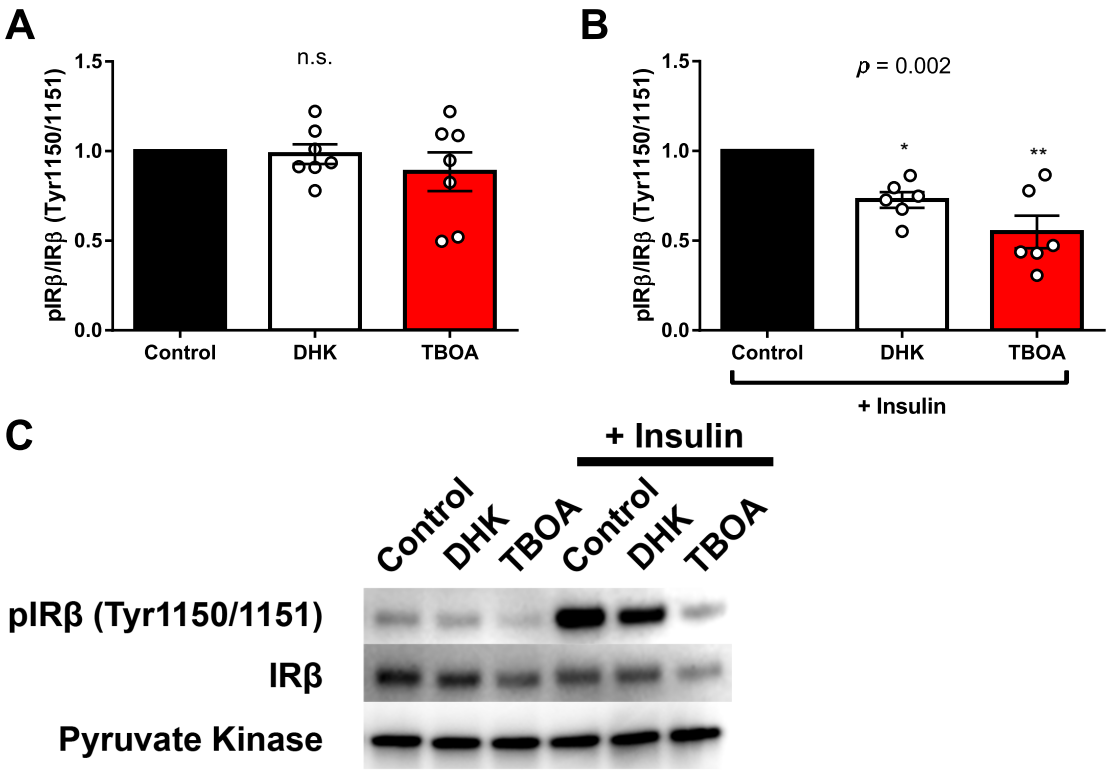

Figure 3.1: Insulin-evoked insulin receptor phosphorylation is reduced by inhibition of neuronal glutamate transporters. (A) Densitometric quantifications of Western blots showed that treatment with DHK or TBOA did not significantly alter the ratio of pIRβ/IRβ ($Tyr^{1150/1151}$) under basal conditions (n=7). (B) Under insulin-evoked conditions, however, treatment with either DHK or TBOA resulted in significant (p= 0.002) decreases in the ratio of pIRβ/IRβ (n=7). (C) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001. Data are presented as normalized mean ± SEM.

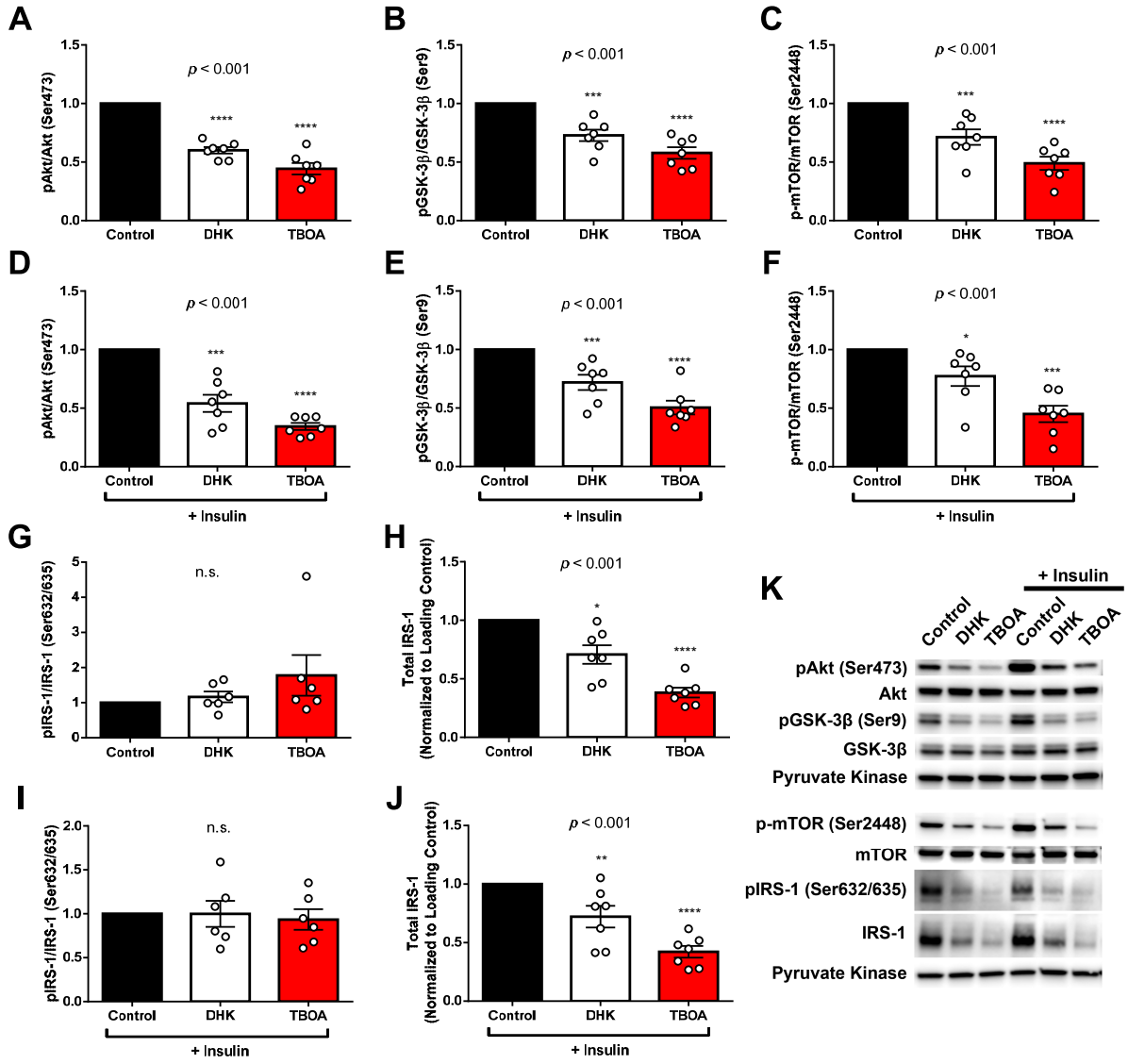

Figure 3.2: Both basal and insulin-evoked downstream insulin signaling are reduced by inhibition of neuronal glutamate transporters. (A-C) Densitometric quantifications of Western blots showed that the levels of (A) pAkt/Akt (Ser^{473}), (B) pGSK-3β/GSK-3β (Ser^{9}), and (C) p-mTOR/mTOR (Ser^{2448}) were all significantly reduced by both DHK and TBOA treatment ($p<$ 0.001 for each) under basal conditions (n=7 for each). (D-F) Similarly, the levels of (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, and (C) p-mTOR/mTOR were all significantly reduced by both DHK and TBOA treatment ($p<$ 0.001 for each) under insulin-evoked conditions (n=7 for each). (G and I) The levels of pIRS-1/IRS-1 ($Ser^{632/635}$) were not significantly altered by DHK or TBOA treatment under either (G) basal or (I) insulin-evoked conditions (n=6 for each). (H and J) However, total levels of IRS-1 (normalized to the loading control pyruvate kinase) were significantly reduced by DHK and TBOA treatment under both (H) basal and (J) insulin-evoked conditions ($p<$ 0.001 and n=7 for each). (K) Representative Western blots are shown. Dunnett's

multiple comparisons test was used to compare the control group to every other group: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001. Data are presented as normalized mean ± SEM**.**

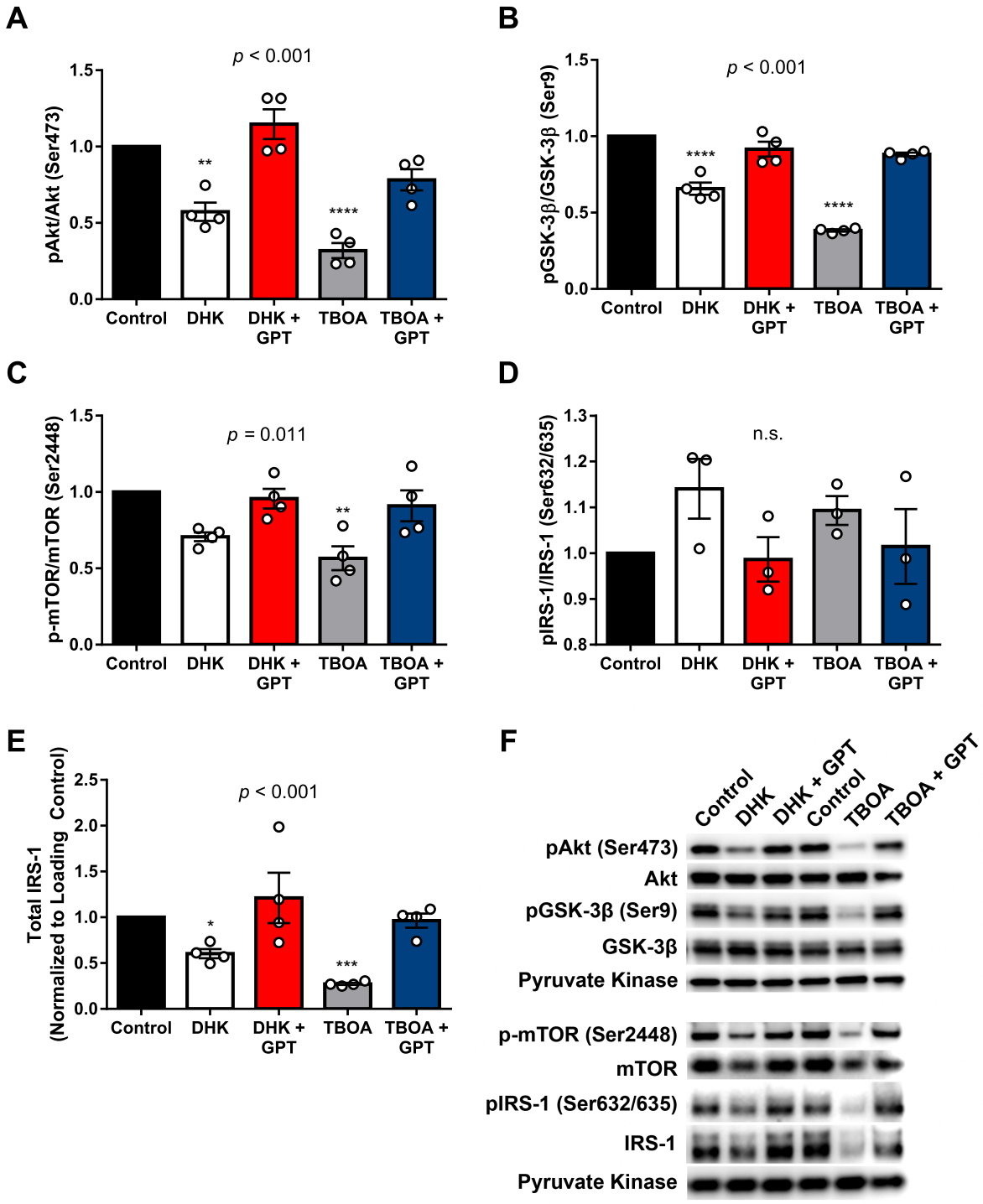
A
p < 0.001
pAkt/Akt (Ser473)
1.5
1.0
0.5
0.0
**
****
Control
DHK
DHK + GPT
TBOA
TBOA + GPT
B
p < 0.001
pGSK-3β/GSK-3β (Ser9)
1.5
1.0
0.5
0.0
****
****
Control
DHK
DHK + GPT
TBOA
TBOA + GPT
C
p = 0.011
p-mTOR/mTOR (Ser2448)
1.5
1.0
0.5
0.0
**
Control
DHK
DHK + GPT
TBOA
TBOA + GPT
D
n.s.
pIRS-1/IRS-1 (Ser632/635)
1.3
1.2
1.1
1.0
0.9
0.8
Control
DHK
DHK + GPT
TBOA
TBOA + GPT
E
p < 0.001
Total IRS-1 (Normalized to Loading Control)
2.5
2.0
1.5
1.0
0.5
0.0
*
***
Control
DHK
DHK + GPT
TBOA
TBOA + GPT
F
Control
DHK
DHK + GPT
Control
TBOA
TBOA + GPT
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.3: Scavenging of extracellular glutamate prevents the insulin signaling changes induced by glutamate transporter inhibition. (A-C, E) Densitometric quantification of Western blots showed, as seen previously, that treatment with DHK and TBOA significantly reduced the levels of (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR (DHK trended toward a decrease but was not significantly different from control by post-hoc analysis), (E) and total IRS-1 ($p< 0.001$; $p< 0.001$; $p= 0.001$; $p< 0.001$; respectively) under basal conditions. However, co-incubation of the glutamate scavenger, glutamic-pyruvic transaminase (GPT; added with pyruvate), with DHK and TBOA maintained (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR, (E) and total IRS-1 at control levels (n=4 for each). (D) The levels of pIRS-1/IRS-1 trended toward an increase due to DHK and TBOA treatment but were not significantly different between groups (n=3). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$. Data are presented as normalized mean ± SEM**.**

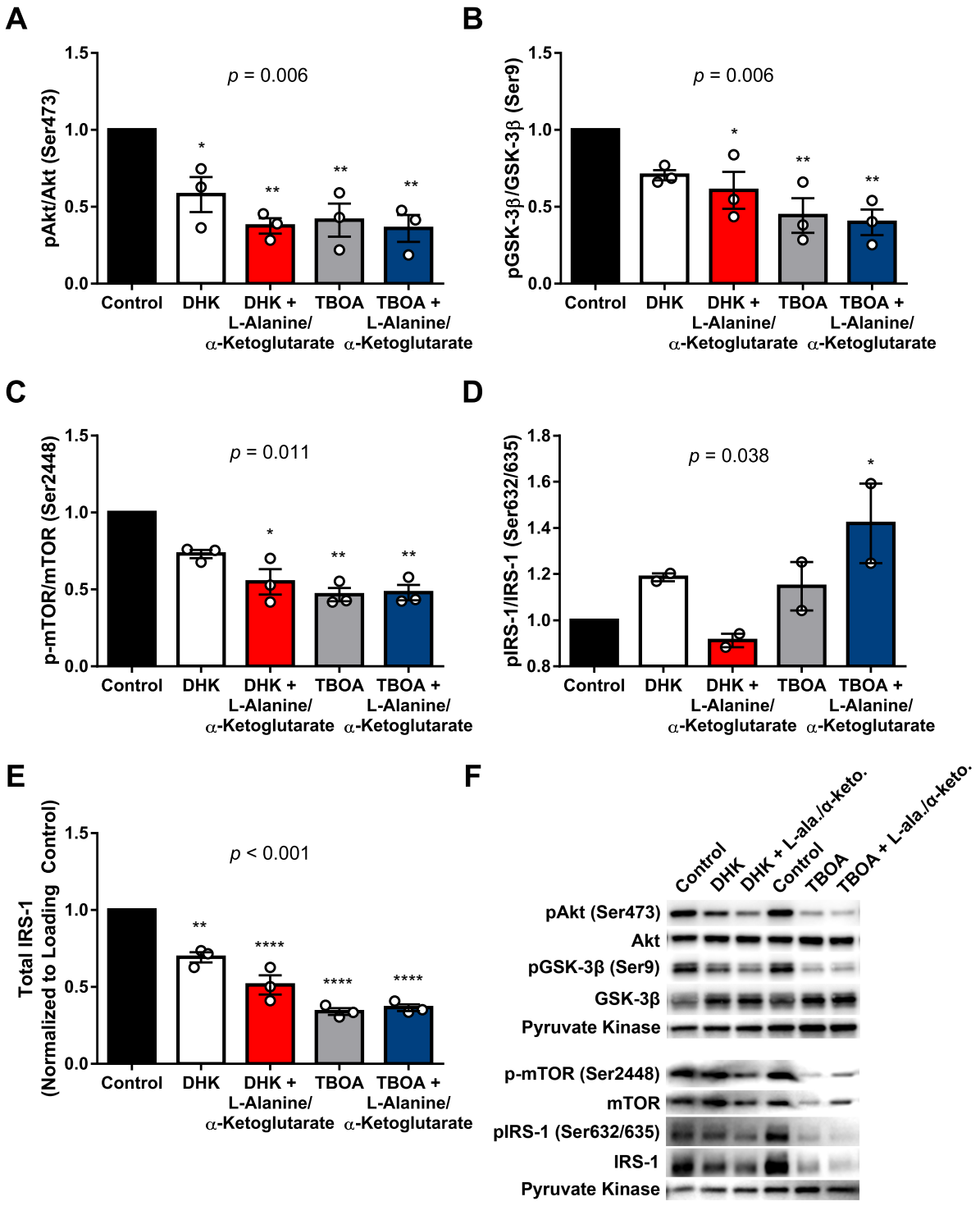
A
pAkt/Akt (Ser473)
p = 0.006
B
pGSK-3β/GSK-3β (Ser9)
p = 0.006
C
p-mTOR/mTOR (Ser2448)
p = 0.011
D
pIRS-1/IRS-1 (Ser632/635)
p = 0.038
E
Total IRS-1 (Normalized to Loading Control)
p < 0.001
Control
DHK
DHK + L-Alanine/ α-Ketoglutarate
TBOA
TBOA + L-Alanine/ α-Ketoglutarate
F
Control
DHK
DHK + L-ala./α-keto.
Control
TBOA
TBOA + L-ala./α-keto.
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.4: The products of glutamate scavenging, L-alanine and α-ketoglutarate, do not prevent insulin signaling changes induced by inhibition of glutamate transport. (A-C, E) Densitometric quantification of Western blots showed that co-treatment with L-alanine and α-ketoglutarate did not prevent the significant reductions in (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR, (E) and total IRS-1 ($p= 0.006$; $p= 0.006$; $p= 0.011$; $p< 0.001$; respectively) induced by DHK and TBOA treatments (n=3 for each; Note: DHK treatment did not reach statistical significance for pGSK-3β/GSK-3β or p-mTOR/mTOR levels although reductions were similar to those identified previously). (D) The levels of pIRS-1/IRS-1 were modestly, but significantly altered ($p= 0.038$), with only the TBOA + L-alanine/α-ketoglutarate group being significantly different from control (n=2). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$. Data are presented as normalized mean ± SEM**.**

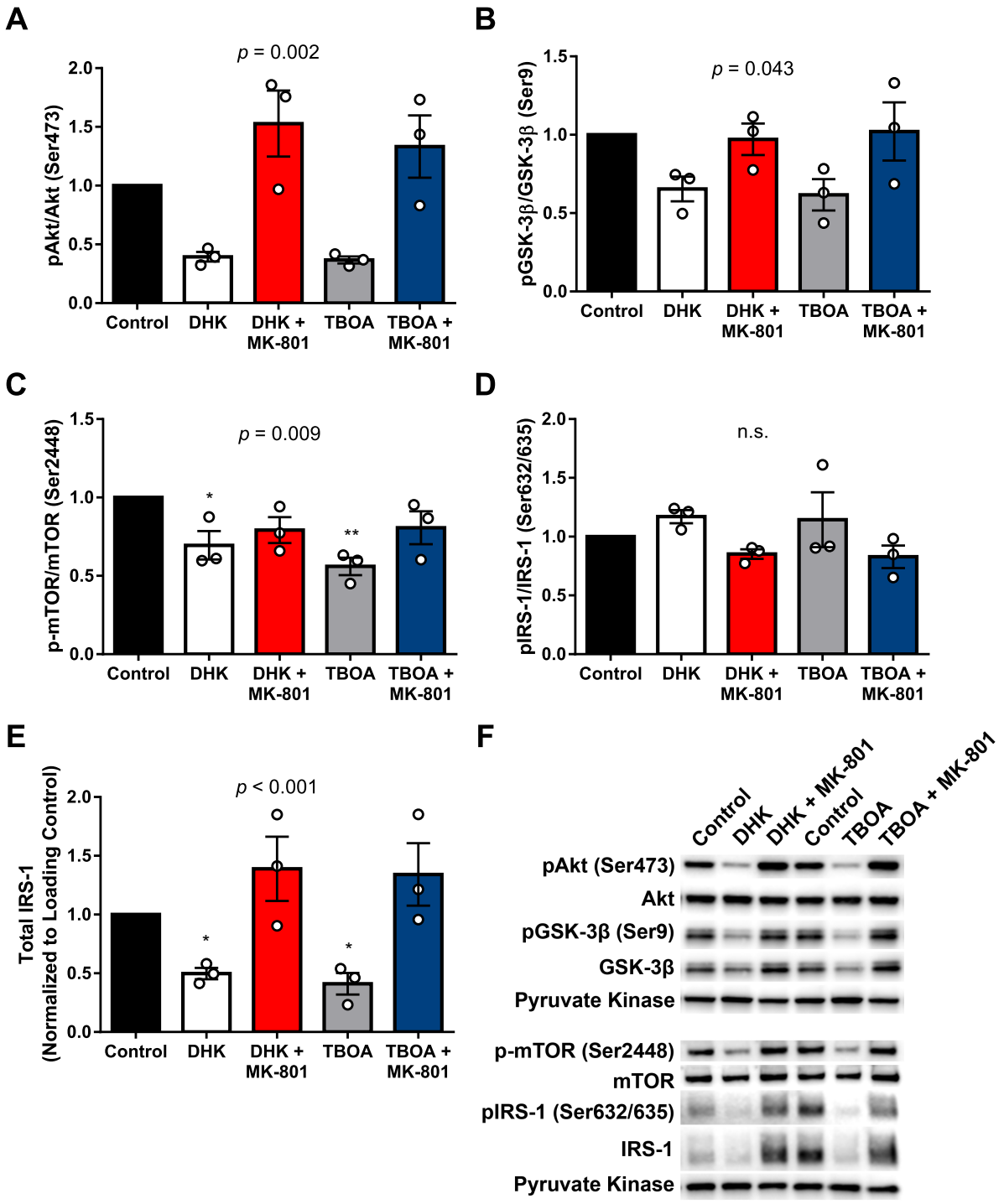
A
p = 0.002
pAkt/Akt (Ser473)
2.0
1.5
1.0
0.5
0.0
Control
DHK
DHK + MK-801
TBOA
TBOA + MK-801
B
p = 0.043
pGSK-3β/GSK-3β (Ser9)
1.5
1.0
0.5
0.0
Control
DHK
DHK + MK-801
TBOA
TBOA + MK-801
C
p = 0.009
p-mTOR/mTOR (Ser2448)
1.5
1.0
0.5
0.0
*
**
Control
DHK
DHK + MK-801
TBOA
TBOA + MK-801
D
n.s.
pIRS-1/IRS-1 (Ser632/635)
2.0
1.5
1.0
0.5
0.0
Control
DHK
DHK + MK-801
TBOA
TBOA + MK-801
E
p < 0.001
Total IRS-1 (Normalized to Loading Control)
2.0
1.5
1.0
0.5
0.0
*
*
Control
DHK
DHK + MK-801
TBOA
TBOA + MK-801
F
Control
DHK
DHK + MK-801
Control
TBOA
TBOA + MK-801
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.5: Insulin signaling alterations induced by glutamate transporter inhibition are prevented by the NMDA receptor antagonist MK-801. (A-C, E) Densitometric quantification of Western blots showed that co-treatment of MK-801 with DHK and TBOA treatments maintained the levels of (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR, (E) and total IRS-1 (p= 0.002; p= 0.043; p= 0.009; p< 0.001; respectively) at control levels (n=3 for each). Although both DHK and TBOA treatments produced similar reductions in insulin signaling as in previous experiments, statistically significant changes were only identified by post-hoc analysis for p-mTOR/mTOR and total IRS-1 levels. (D) The levels of pIRS-1/IRS-1 trended toward an increase due to DHK and TBOA treatments but were not significantly different between groups (n=3). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001. Data are presented as normalized mean ± SEM**.**

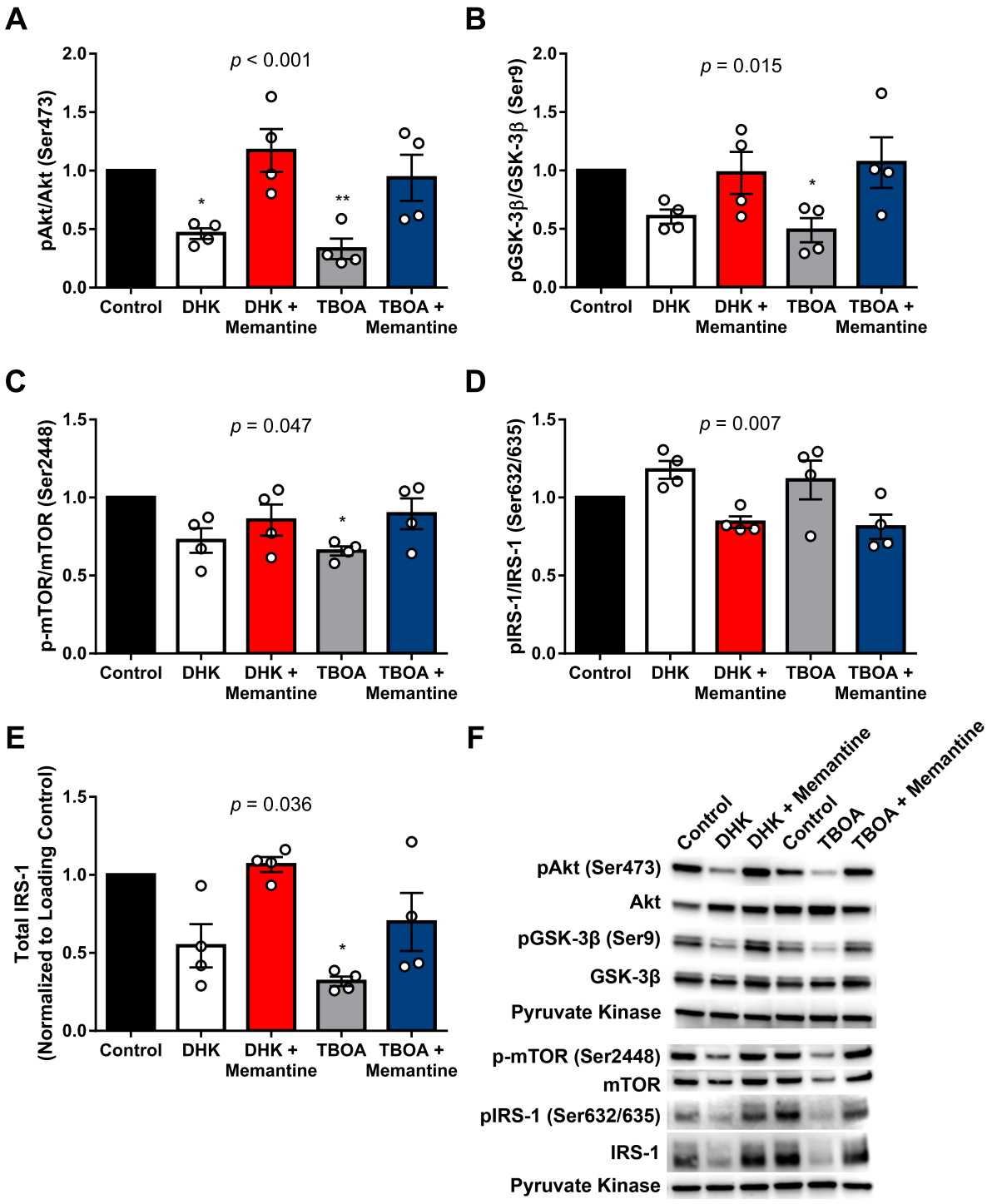
A
pAkt/Akt (Ser473)
p < 0.001
2.0
1.5
1.0
0.5
0.0
*
**
Control
DHK
DHK + Memantine
TBOA
TBOA + Memantine
B
pGSK-3β/GSK-3β (Ser9)
p = 0.015
*
C
p-mTOR/mTOR (Ser2448)
p = 0.047
*
D
pIRS-1/IRS-1 (Ser632/635)
p = 0.007
E
Total IRS-1 (Normalized to Loading Control)
p = 0.036
*
F
Control
DHK
DHK + Memantine
Control
TBOA
TBOA + Memantine
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.6: Insulin signaling alterations induced by glutamate transporter inhibition are also prevented by the NMDA receptor antagonist memantine. (A-C, E) Densitometric quantification of Western blots showed that co-treatment of memantine with DHK and TBOA maintained the levels of (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR, (E) and total IRS-1 ($p< 0.001$; $p= 0.015$; $p= 0.047$; $p= 0.036$; respectively) at control levels (n=4 for each). Both DHK and TBOA treatments produced similar reductions in insulin signaling, but DHK treatment only reached statistical significance by post-hoc analysis for pAkt/Akt. (D) The levels of pIRS-1/IRS-1 were significantly different between groups with a trend toward an increase due to DHK and TBOA treatments, but no significant differences between any group and control were identified by post-hoc analysis (n=4). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$. Data are presented as normalized mean ± SEM**.**

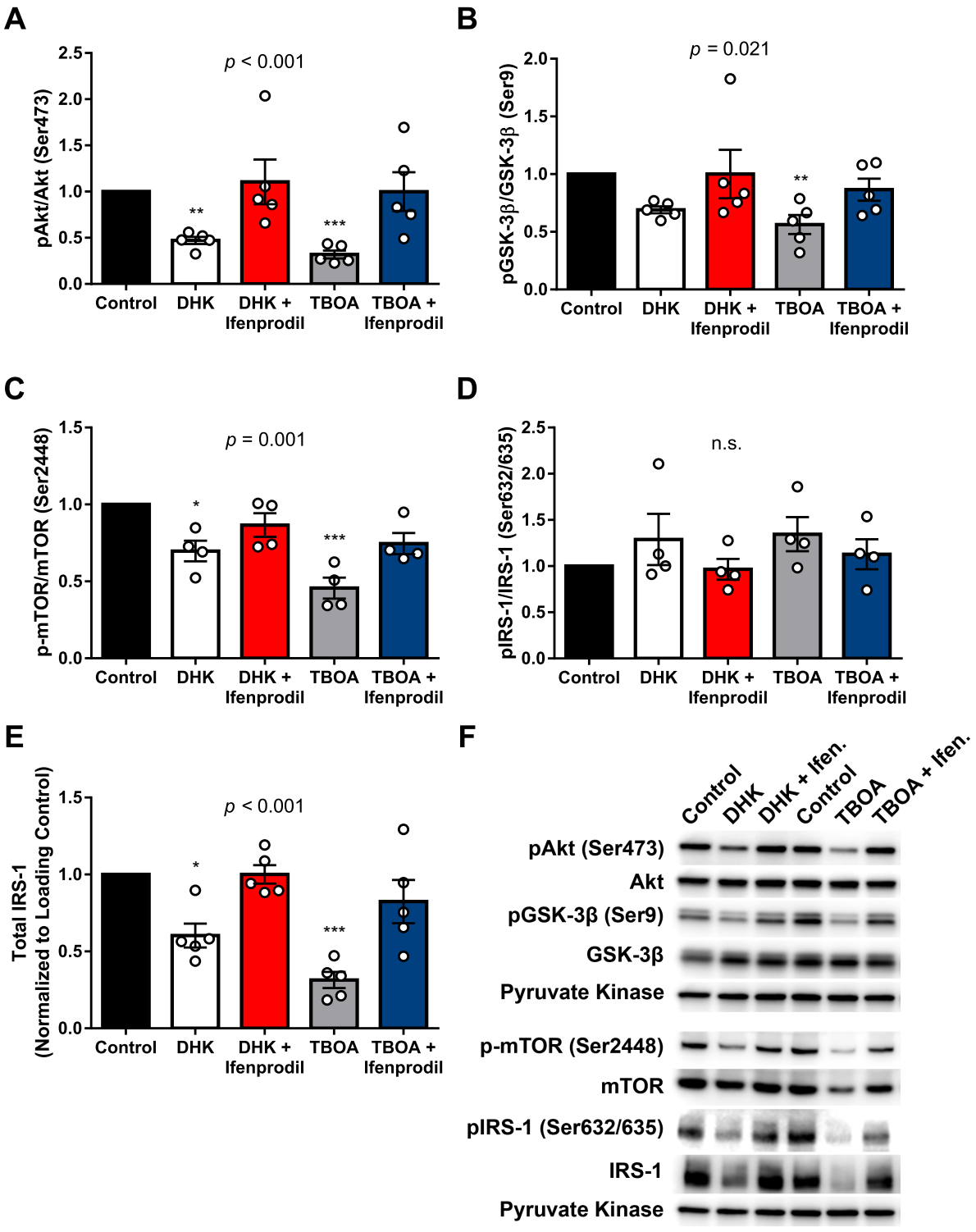
A
pAkt/Akt (Ser473)
p < 0.001
**
***
Control
DHK
DHK + Ifenprodil
TBOA
TBOA + Ifenprodil
B
pGSK-3β/GSK-3β (Ser9)
p = 0.021
**
C
p-mTOR/mTOR (Ser2448)
p = 0.001
*
***
D
pIRS-1/IRS-1 (Ser632/635)
n.s.
E
Total IRS-1 (Normalized to Loading Control)
p < 0.001
*
***
F
Control
DHK
DHK + Ifen.
Control
TBOA
TBOA + Ifen.
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.7: The NR2B-selective NMDA receptor antagonist ifenprodil also prevents insulin signaling alterations induced by inhibition of glutamate transport. (A-C, E) Densitometric quantifications of Western blots showed that co-treatment of ifenprodil with DHK and TBOA maintained the levels of (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR, (E) and total IRS-1 at control levels while treatment with DHK and TBOA alone caused significant reductions in (A) pAkt/Akt, (B) pGSK-3β/GSK-3β (TBOA only), (C) p-mTOR/mTOR, (E) and total IRS-1 ($p< 0.001$; $p= 0.021$; $p= 0.001$; $p< 0.001$; respectively; n=4-5 for each). (D) The levels of pIRS-1/IRS-1 again trended toward a slight increase due to DHK and TBOA treatments but were not significantly different between groups (n=4). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: $*p< 0.05$, $**p< 0.01$, $***p< 0.001$, $****p< 0.0001$. Data are presented as normalized mean ± SEM**.**

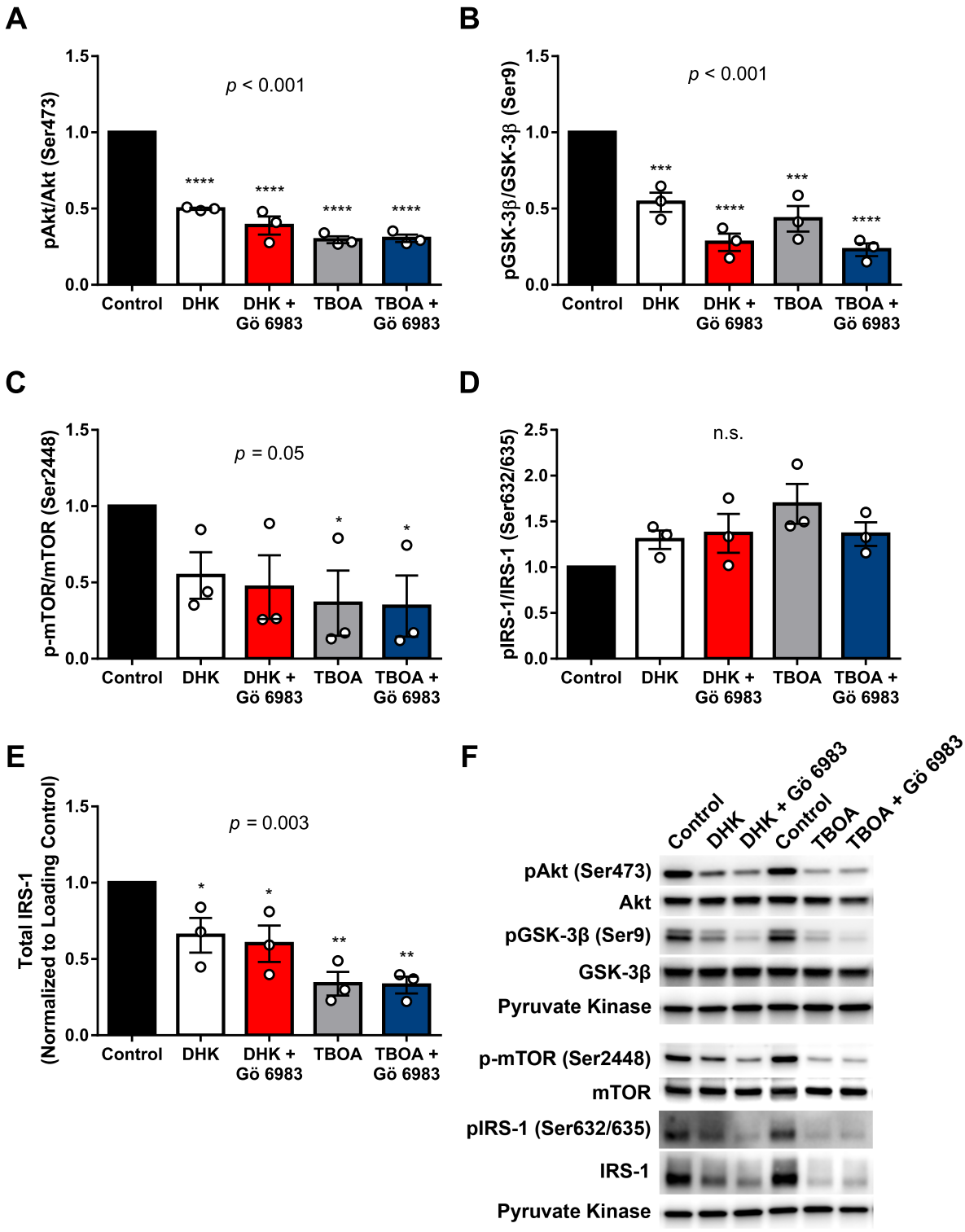
A
pAkt/Akt (Ser473)
p < 0.001
****
Control
DHK
DHK + Gö 6983
TBOA
TBOA + Gö 6983
B
pGSK-3β/GSK-3β (Ser9)
p < 0.001
***
****
C
p-mTOR/mTOR (Ser2448)
p = 0.05
*
D
pIRS-1/IRS-1 (Ser632/635)
n.s.
E
Total IRS-1
(Normalized to Loading Control)
p = 0.003
**
F
Control
DHK
DHK + Gö 6983
Control
TBOA
TBOA + Gö 6983
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.8: Inhibition of protein kinase C (PKC) does not prevent insulin signaling alterations induced by inhibition of glutamate transport. (A-C, E) Densitometric quantifications of Western blots showed that DHK and TBOA treatments caused significant reductions in the levels of (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR, (E) and total IRS-1 whether or not the PKC inhibitor Gö 6983 was present ($p< 0.001$; $p< 0.001$; $p= 0.05$; $p= 0.003$; respectively; n=3 for each). The reductions in p-mTOR/mTOR induced by DHK and DHK + Gö 6983 did not reach statistical significance by post-hoc analysis. (D) The levels of pIRS-1/IRS-1 trended toward a slight increase due to DHK and TBOA treatments, with or without Gö 6983, but were not significantly different between groups (n=3). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$. Data are presented as normalized mean ± SEM**.**

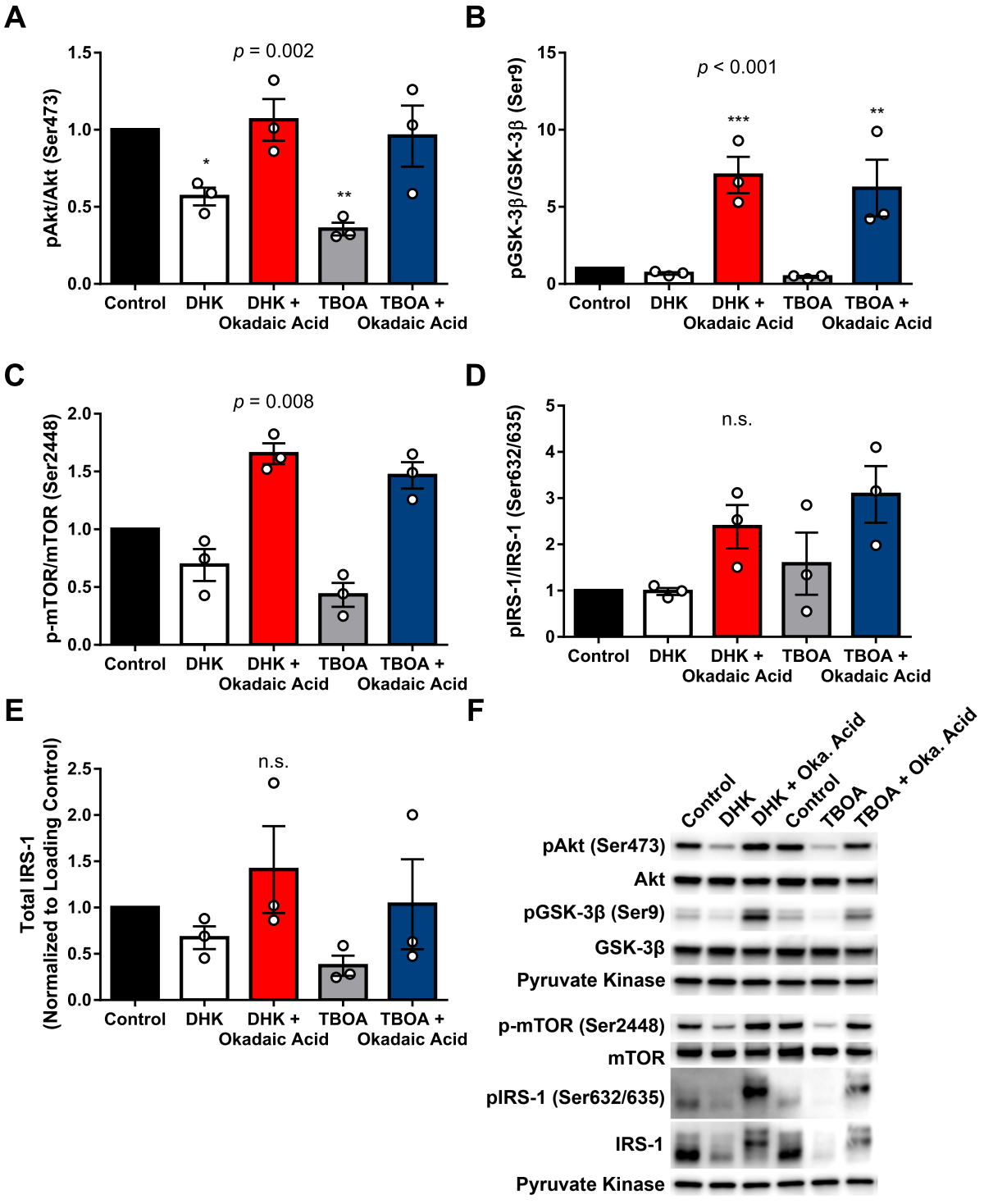
A
p = 0.002
pAkt/Akt (Ser473)
1.5
1.0
0.5
0.0
*
**
Control
DHK
DHK + Okadaic Acid
TBOA
TBOA + Okadaic Acid
B
p < 0.001
pGSK-3β/GSK-3β (Ser9)
15
10
5
0
***
**
C
p = 0.008
p-mTOR/mTOR (Ser2448)
2.0
1.5
1.0
0.5
0.0
D
n.s.
pIRS-1/IRS-1 (Ser632/635)
5
4
3
2
1
0
E
n.s.
Total IRS-1 (Normalized to Loading Control)
2.5
2.0
1.5
1.0
0.5
0.0
F
Control
DHK
DHK + Oka. Acid
Control
TBOA
TBOA + Oka. Acid
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.9: Inhibition of protein phosphatases 1 and 2A (PP1/PP2A) prevents alterations to Akt induced by glutamate transporter inhibition but differentially regulates other insulin signaling proteins. (A) Densitometric quantifications of Western blots showed that DHK and TBOA treatments caused significant reductions in the levels of pAkt/Akt, which were prevented by co-incubation with the PP1/PP2A inhibitor okadaic acid (p= 0.002; n=3). (B, C, and E) However, okadaic acid treatment with DHK and TBOA also dramatically increased the levels of (B) pGSK-3β/GSK-3β (p< 0.001; n=3) with similar trends observed for the levels of (C) p-mTOR/mTOR (p= 0.008; n=3) and (E) and total IRS-1 (n.s.; n=3). (D) Okadaic acid treatment with DHK and TBOA also produced a strong trend for increased levels of pIRS-1/IRS-1 although there was not a significant difference between groups (n=4). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001. Data are presented as normalized mean ± SEM**.**

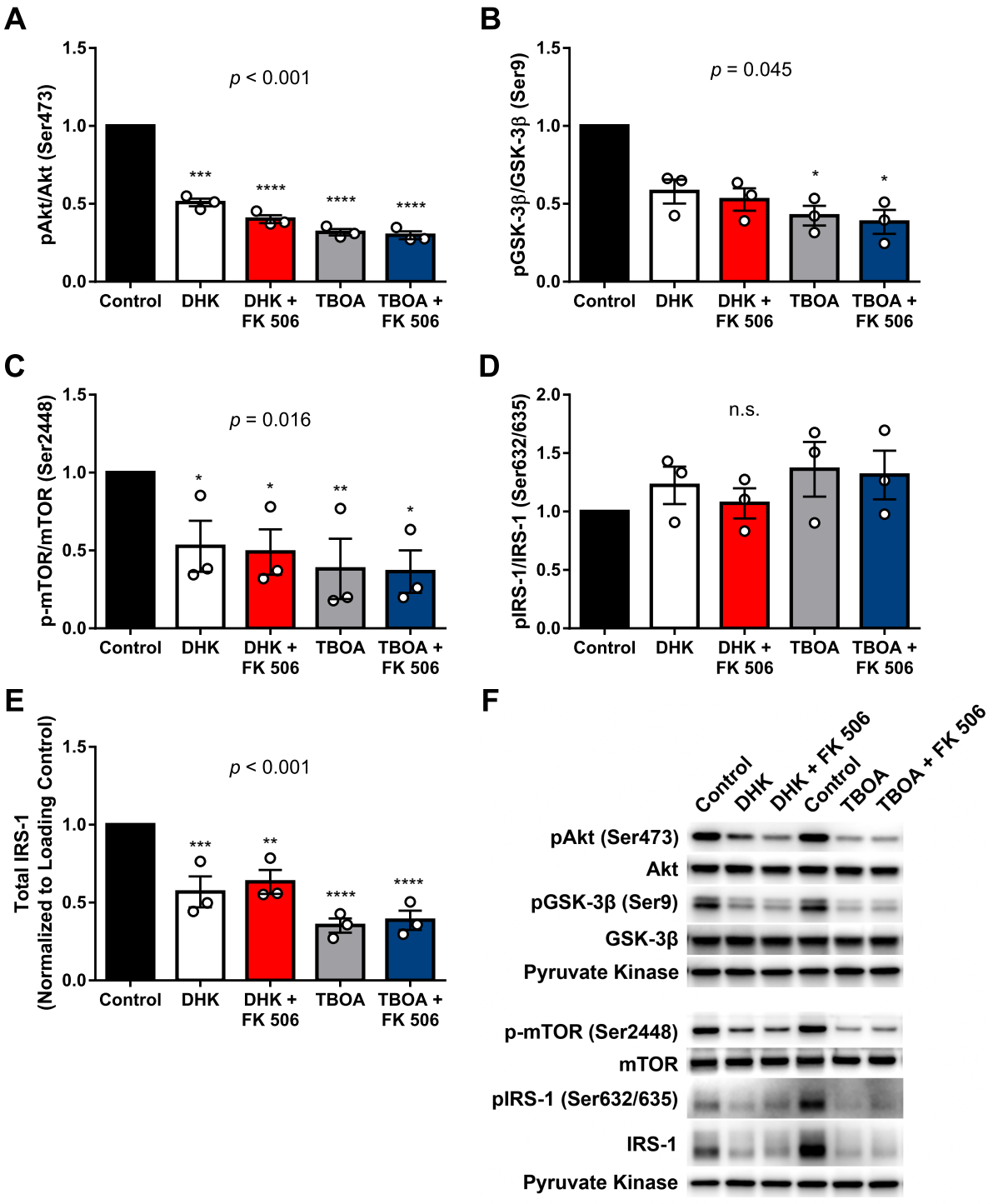
A
pAkt/Akt (Ser473)
p < 0.001
***
****
****
****
B
pGSK-3β/GSK-3β (Ser9)
p = 0.045
*
*
C
p-mTOR/mTOR (Ser2448)
p = 0.016
*
*
**
*
D
pIRS-1/IRS-1 (Ser632/635)
n.s.
E
Total IRS-1
(Normalized to Loading Control)
p < 0.001
***
**
****
****
Control
DHK
DHK + FK 506
TBOA
TBOA + FK 506
F
Control
DHK
DHK + FK 506
Control
TBOA
TBOA + FK 506
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Pyruvate Kinase
p-mTOR (Ser2448)
mTOR
pIRS-1 (Ser632/635)
IRS-1
Pyruvate Kinase

Figure 3.10: Inhibition of protein phosphatase 2B (PP2B) does not prevent insulin signaling alterations induced by inhibition of glutamate transport. (A-C, E) Densitometric quantifications of Western blots showed that DHK and TBOA treatments caused significant reductions in the levels of (A) pAkt/Akt, (B) pGSK-3β/GSK-3β, (C) p-mTOR/mTOR, (E) and total IRS-1 whether or not the PP2B inhibitor FK 506 was present ($p< 0.001$; $p= 0.045$; $p= 0.016$; $p< 0.001$; respectively; n=3 for each). The reductions in pGSK-3β/GSK-3β induced by DHK and DHK + FK 506 did not reach statistical significance by post-hoc analysis. (D) The levels of pIRS-1/IRS-1 trended toward a slight increase due to DHK and TBOA treatments, with or without Gö 6983, but were not significantly different between groups (n=3). (F) Representative Western blots are shown. Dunnett's multiple comparisons test was used to compare the control group to every other group: *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$. Data are presented as normalized mean ± SEM**.**

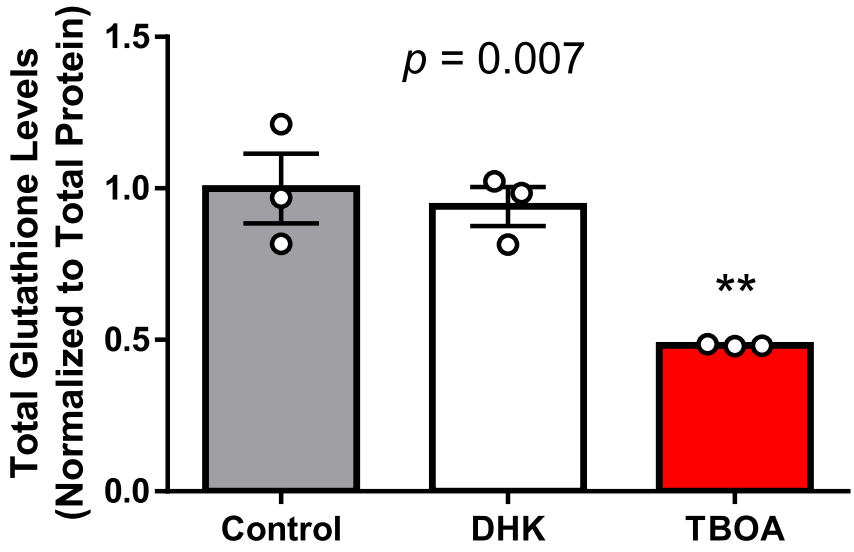

Figure 3.11: Total glutathione levels are reduced by inhibition of GLT-1 and EAAC1, but not GLT-1 alone. Total glutathione levels were measured in neuronal lysates. Treatment with TBOA caused a significant reduction in total glutathione levels while treatment with DHK did not significantly alter total glutathione content ($p < 0.007$; n=3). Dunnett's multiple comparisons test was used to compare the control group to every other group: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data are presented as normalized mean ± SEM**.**

CHAPTER 4:

Conclusions

AD is a progressive neurodegenerative disease that currently affects millions. Despite intensive research efforts, much remains to be understood about the pathogenesis of AD. In the previous chapters, I have reviewed the current evidence for roles of both glutamatergic signaling dysfunction and insulin signaling disturbances in AD pathogenesis and provided further evidence that links these two previously distinct pathologies.

Earlier work from our lab (Mookherjee et al., 2011) had shown that partial loss of GLT-1 leads to early-occurring cognitive deficits in mice expressing familial AD mutations (APPswe/PS1ΔE9). However, other than a modest effect on the ratio of Aβ42/Aβ40 levels, partial GLT-1 loss did not significantly alter amyloid pathology suggesting that changes to amyloid were not responsible for the GLT-1 loss-induced cognitive dysfunction. Given that GLT-1 expression is influenced by insulin signaling, that both glutamate and insulin signaling play important roles in memory and cognitive processes, and that dysfunction of each is implicated in AD, I hypothesized that partial GLT-1 loss might lead to insulin signaling disturbances in this AD mouse model (Meeker et al., 2015). Alterations were identified in the phosphorylation states of several components of the CNS insulin signaling cascade, which included: decreased insulin receptor phosphorylation ($Tyr^{1150/1151}$), increased Akt phosphorylation (Ser^{473}), and increased IRS-1 phosphorylation ($Ser^{636/639}$). These insulin signaling changes are very similar to those found in AD brains, indicative of an overall reduction in insulin signaling possibly through negative feedback by activated Akt. This has been postulated to be a form of brain insulin resistance by others (Talbot et al., 2012).

One important point to consider is that the insulin signaling changes that resulted from partial GLT-1 loss occurred early in the course of pathology, mimicking the onset of cognitive deficits observed in these mice. Moreover, differences in insulin signaling between groups became blunted over time similar to the differences in cognition. In AD, other studies have shown that both GLT-1 loss and insulin signaling changes both occur early in the course of AD, with disturbances evident even in individuals with mild cognitive impairment (MCI) (Talbot et al., 2012; Woltjer et al., 2010). Taken together, this suggests that loss of GLT-1 early in the course of AD may result in insulin signaling disturbances and cognitive impairment. Given that both glutamatergic and insulin signaling are important for learning and memory, it seems likely that disturbances to both of these pathways would result in negative consequences to cognition. This early loss of GLT-1 expression and function may be due to Aβ as it has been found to oxidatively damage GLT-1 (Lauderback et al., 2001). Aβ oligomers have also been found to interfere with insulin signaling suggesting that disturbances to each of these pathways in AD may be two-fold: first through dysfunction induced by Aβ and secondly through signaling interactions between GLT-1 loss and the insulin signaling pathway.

These *in vivo* findings show that GLT-1 loss results in alterations in the phosphorylation states of several key proteins in the insulin signaling cascade, which as mentioned above, have been shown in several other studies to be disturbed in AD. The majority of the data in AD patients implicates changes specifically in neuronal insulin signaling, yet GLT-1 expression is highest in astrocytes. This raises the possibility that my findings reflect astrocyte-mediated changes that are manifested by neurons. It has recently been established, however, that GLT-1 is also expressed in neuronal axon terminals. While neuronal expression of GLT-1 only accounts for roughly 10% or less of total GLT-1 in the brain, it has been found to be responsible for an

equivalent amount of uptake as that in astrocytes (Furness et al., 2008). Furthermore, recent evidence suggests that neuronal GLT-1 is responsible for a much greater percentage of synaptosomal uptake, while astrocytic GLT-1 is critical for preventing broad excitotoxicity and epileptic events (Petr et al., 2015), potentially suggesting that neuronal GLT-1 may play a role in modulating local glutamatergic signaling near synapses. Thus, we sought to determine if loss of neuronal GLT-1 contributed to the resulting insulin signaling alterations seen *in vivo*.

Using purified primary cortical neuron cultures, we found that loss of GLT-1 function (through pharmacological inhibition) resulted in alterations in insulin signaling under both insulin-evoked and basal conditions. These changes included decreased insulin receptor phosphorylation ($Tyr^{1150/1151}$; only under insulin-evoked conditions), Akt phosphorylation (Ser^{473}), GSK-3β phosphorylation (Ser^{9}), mTOR phosphorylation (Ser^{2448}), and total IRS-1 levels. While the reductions in the phosphorylation states of each of these proteins (and total IRS-1) are consistent with each other and are indicative of decreased insulin signaling, these changes are in a distinct pattern from what was observed *in vivo*. This can be noted by the differences in the phosphorylation of downstream proteins such as Akt, GSK-3β, and mTOR. For example, Akt phosphorylation increased due to GLT-1 and amyloid pathology *in vivo* while it was decreased due to GLT-1 inhibition *in vitro*, suggesting negative feedback regulation of insulin signaling *in vivo* but not *in vitro*. As discussed earlier, these differences are likely due to interactions of other factors and pathways that are influencing the basal activation states *in vivo* but not *in vitro*. This is best illustrated by insulin signaling changes identified in human brain slices, where the activation states of proteins at the top of the pathway (i.e. insulin receptor, IRS-1) are reduced while the activation states of downstream proteins (i.e. Akt, GSK-3β, mTOR) are unchanged or increased compared to controls under basal conditions. However, when the slices

are treated with insulin, providing direct stimulation of the pathway, the activation states of all insulin signaling proteins are reduced compared to controls (Talbot et al., 2012).

While collectively, the *in vivo* and *in vitro* results suggest that GLT-1 loss or dysfunction promotes a state of decreased insulin signaling, the mechanism of how this interaction was occurring still needed to be identified. Two potential mechanisms emerged for how this could be occurring: (i) loss of GLT-1 function results in increased extracellular glutamate levels, increased signaling through glutamate receptors, and resulting insulin signaling changes; or (ii) loss of GLT-1 function prevents uptake of cysteine in neurons, leading to reduced formation of the antioxidant glutathione and increased oxidative stress thereby resulting in insulin signaling changes. In support of the former, both scavenging of extracellular glutamate and inhibition of NMDA-type glutamate receptor activation prevented the insulin signaling changes induced by GLT-1 inhibition. While those results on their own do not rule out the latter hypothesis, it was found that inhibition of GLT-1 alone did not result in significant changes to total glutathione levels. Together, these results suggest that disturbances to glutamatergic signaling alone are sufficient to cause insulin signaling disturbances after GLT-1 loss. In particular, it should be noted that GluN2B-containing NMDA receptors, which may be located extrasynaptically, seemed to be responsible for the effects of GLT-1 loss. This may be due to their localization in regions where glutamate is not normally able to diffuse when transporter function is normal, or potentially to differences in activation kinetics of this particular type of NMDA receptor composition. Furthermore, while we attempted to determine the intracellular signals linking NMDA receptor activation to the resulting insulin signaling downregulation, a clear link was not determined. Thus, further studies will be required to determine how NMDA receptor activation after GLT-1 loss causes changes in insulin signaling.

In conjunction with examining the effect of neuronal GLT-1 loss on insulin signaling *in vitro*, we also examined changes induced by inhibition of both neuronal glutamate transporters, GLT-1 and EAAC1. From this, it was found that inhibition of both transporters led to similar, but often slightly greater decreases, in the same insulin signaling proteins. This suggests that EAAC1 may play a similar role as GLT-1 in regulating insulin signaling through modulation of extracellular glutamate concentration. This may explain why GLT-1 and EAAC1 exist in distinct subcellular regions of neurons, with GLT-1 located presynaptically and on axons while EAAC1 is present on dendrites and the cell soma. Interestingly, while inhibition of GLT-1 alone did not significantly alter total glutathione levels, blockade of both GLT-1 and EAAC1 together resulted in a significant decrease in total glutathione. This may suggest that EAAC1 plays a more prominent role in cysteine uptake than GLT-1 in neurons, although blockade of both transporters together may synergistically affect glutathione synthesis. However, this may also suggest that some of the more pronounced insulin signaling effects induced by inhibition of both transporters could be due to changes in glutamatergic signaling and the oxidative state of the neurons. Further studies are needed to address this point.

The research presented in this thesis has led to several novel findings: (i) Partial loss of GLT-1 leads to disturbances in brain insulin signaling *in vivo* in an animal model of AD, which mirror cognitive deficits previously identified in these mice; (ii) the insulin signaling disturbances identified *in vivo* accompanying partial GLT-1 loss closely mimic CNS insulin signaling defects found in AD patients; and (iii) dysfunction of neuronal GLT-1 *in vitro* leads to accumulation of extracellular glutamate and activation of NMDA receptors resulting in similar disturbances to insulin signaling. To our knowledge, these data are the first *in vivo* demonstration

of GLT-1 loss altering insulin signaling and the first report of a functional role for neuronally expressed GLT-1.

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