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Partial Loss of the Glutamate Transporter GLT-1 Alters Brain Akt and Insulin Signaling in a Mouse Model of Alzheimer's Disease

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Abstract

The glutamate transporter GLT-1 (also called EAAT2 in humans) plays a critical role in regulating extracellular glutamate levels in the central nervous system (CNS). In Alzheimer's disease (AD), EAAT2 loss is associated with 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 A β PPswe/PS1 E9 mutations. GLT-1 plays important roles in several molecular pathways that regulate brain metabolism, including Akt and insulin signaling in astrocytes. 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 A β PPswe/PS1 E9 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 serine 473), impaired insulin signaling (reflected by decreased IR β phosphorylation of tyrosines 1150/1151 and increased IRS-1 phosphorylation at serines 632/635 – denoted as 636/639 in humans), and reduced insulin degrading enzyme (IDE) activity in brains of mice expressing familial A β PPswe/PS1 E9 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 amyloid- β levels and expresses GLT-1. Taken together, these findings demonstrate that partial GLT-1 loss can cause insulin/Akt signaling abnormalities that are in keeping with those observed in AD.

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Keywords

Amyloid- β ; EAAT2; excitotoxicity; GLT1; metabolism; SLC1A2

INTRODUCTION

The amino acid glutamate plays a crucial role in excitatory neurotransmission in the central nervous system (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 [1–3]. 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 80–90% of the released extracellular glutamate [1, 2, 4, 5]. The biological significance of GLT-1 is underscored by findings that GLT-1 knockout (–/–) mice develop lethal seizures accompanied by significant hippocampal neuron loss [1]. GLAST, EAAC1, and EAAT4 deficiency are also functionally significant. However, these knockout mice display less severe phenotypes than GLT-1 deficient mice [6–9].

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 Huntington's disease [10, 11], traumatic brain injury [12], and amyotrophic lateral sclerosis [13–15]. A number of laboratories have also recently reported that EAAT2 is significantly reduced in AD by as much as 50% [16–19].

In a previous study, we reported that GLT-1 heterozygous (GLT-1+/-) mice harboring compound familial AD mutations of the amyloid- β protein precursor (A β PPswe) and Presenilin 1 (PS1 E9) displayed early-occurring cognitive deficits compared to A β PPswe/PS1 E9 mice with normal wild-type GLT-1 (GLT-1+/+) levels [20]. These findings demonstrate that partial GLT-1 loss is capable of unmasking early-occurring cognitive disturbances brought about by expression of the AD-related A β PPswe/PS1 E9 transgene [20].

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 β ₄₂/A β ₄₀ in GLT-1 (+/-)/A β PPswe/PS1 E9 mice, total A β PP levels and amyloid plaque levels were not affected by GLT-1 heterozygosity [20]. Thus, amyloid plaque burden appears to be a less likely explanation for the behavioral impairments exhibited by GLT-1 (+/-)/A β PPswe/PS1 E9 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 [1]. While A β ₄₂ exposure significantly slows the rate at which GLT-1 clears synaptically released glutamate [21], thereby increasing glutamate spread, we have recently shown that it

does not prevent extracellular glutamate concentrations from returning to basal levels that are estimated to be in the low nanomolar range [22]. Such findings argue against the idea that the consequences of A β -mediated GLT-1 loss are due to chronic basal changes in extracellular glutamate concentrations.

In addition to the importance of GLT-1 in protecting against glutamate-induced toxicity [1–3], 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 [23, 24] and GLT-1 expression is regulated by Akt and insulin stimulation [25–27]. 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 [28–34].

Such findings prompted us to test the hypothesis that partial GLT-1 loss in A β PPswe/PS1 E9 mice would alter Akt and insulin-sensitive 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 (IR β) 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 [28, 31, 35–38].

MATERIALS AND METHODS

Animals

A β PPswe/PS1 E9 (A β PP/PS1) hemizygous mice (line 85) maintained on a B6C3F1/J background [39] and GLT-1 heterozygous (GLT_{het}) mice maintained on a C57BL/6 background [1] were mated in order to generate F₁ offspring littermates with the genotypes: GLT-1_{wt}/non-transgenic (GLT_{wt}/nTg), GLT-1_{het}/non-transgenic (GLT_{het}/nTg), GLT-1_{wt}/A β PPswe/PS1 E9 (GLT_{wt}/A β PP/PS1), and GLT-1_{het}/A β PPswe/PS1 E9 (GLT_{het}/A β PP/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 [20]. 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 $\times g$ 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, phospho-IRS-1/IRS-1, phospho-mTOR/mTOR, phospho-p70 S6 kinase/p70 S6 kinase, and IDE, the 20,000 $\times g$ supernatant was further centrifuged at approximately 135,000 $\times g$ for 1 h at 4°C. The resulting supernatant was used as the soluble fraction. For the crude membrane fraction, to analyze phospho-IR β /IR β , the 20,000 $\times g$ pellet was resuspended in lysis buffer containing 1% Triton X-100, briefly

sonicated, and centrifuged at $20,000\times g$ 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 (pIR β , Tyr^{1150/1151}), total insulin receptor β -subunit (IR β), phospho-Akt (pAkt, Ser⁴⁷³), 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, Ser²⁴⁴⁸), total mTOR, phospho-p70 S6 kinase (p-p70 S6K, Thr³⁸⁹), 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 IR β and IDE, which were obtained from Millipore, 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 enhanced chemiluminescence (ECL) (GE Healthcare, Piscataway, NJ) and an ImageQuant LAS 4000 (GE Healthcare). Densitometry analyses were performed using ImageQuant TL software (GE Healthcare). Band intensities of phosphorylated proteins were normalized to band intensities of corresponding total protein levels. For each protein examined (Akt, IR β , 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 [37–40]. 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 [¹²⁵I]-insulin (Phoenix Pharmaceuticals, Inc., Burlingame, CA) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 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% TCA. After chilling for 15 min at 4°C , the samples were centrifuged at approximately $15,000\times g$ 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 $\pm$ standard error of the mean (SEM) and analyzed using standard analysis of variance (ANOVA) methods. Planned comparison Helmert tests [41–42] were performed upon a statistically significant ANOVA result ($p < 0.05$) to test the *a priori* hypothesis that wild-type, non-transgenic mice would be different from GLT-1 heterozygous and transgenic A β PPswe/PS1 E9 mice. Statistical analyses were carried out using SPSS software (IBM, Armonk, NY).

RESULTS

Sustained Akt activation, as reflected by increased levels of phosphorylation at Ser⁴⁷³, has been reported in brains of AD patients [28–30–32]. To examine whether partial GLT-1 loss in the context of AD-related pathology alters CNS Akt signaling *in vivo*, we measured phospho-Akt (Ser⁴⁷³) 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}/A β PP/PS1; and (iv) GLT_{het}/A β PP/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 β ₄₂/A β ₄₀ at 6, but not at 9 months of age [20].

GLT-1 heterozygosity and A β PP/PS1 transgene expression activate Akt

At 6 months (Fig. 1A, C) we found a statistically significant overall difference among genotypes in the ratios of phospho-Akt (Ser⁴⁷³) to total Akt protein ($F[3,18] = 5.666$, $p < 0.006$). A Helmert specific comparisons test [41–42] confirmed that phospho-Akt/Akt ratios were significantly lower in GLT_{wt}/nTg mice compared to the other three groups ($p < 0.001$). Similarly, at 9 months (Fig. 1B, D) there was a statistically significant overall difference among genotypes in phospho-Akt/Akt ratios ($F[3,20] = 9.551$, $p < 0.001$). 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 ($p < 0.004$). However, at 9 months this difference in Akt phosphorylation among the GLT_{het}/nTg and GLT_{wt}/A β PP/PS1 groups was not as pronounced as at 6 months (Fig. 1B, D).

GLT-1 loss or A β PP/PS1 transgene expression suppresses insulin receptor activation

The findings in Fig. 1 demonstrate that GLT-1 heterozygosity and A β PPswe/PS1 E9 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 [43–45]. In order to test whether sustained Akt elevation due to GLT-1 heterozygosity and/or A β PP/PS1 expression would be accompanied by reduced insulin receptor phosphorylation, we measured the levels of phosphorylated insulin receptor β -subunit (IR β -Tyr^{1150/1151}) and total IR β by western blot analysis.

At 6 months (Fig. 2A, C) we found an overall difference among genotypes in the ratio of phospho-IR β (Tyr^{1150/1151}) to total IR β protein ($F[3,20] = 3.374$, $p = 0.039$). A Helmert analysis supported the conclusion that both GLT-1 heterozygosity and A β PP/PS1 transgene expression were sufficient to significantly suppress IR β phosphorylation in all three groups

compared to GLT_{wt}/nTg mice ($p = 0.005$). However, at 9 months there was no statistically significant difference among groups in the ratios of phospho-IR β (Tyr^{1150/1151})/IR β ($F[3,20] = 2.042$, n.s.). These findings indicate that at 6 months (a time point at which behavioral and A β ₄₂/A β ₄₀ abnormalities were most prominent [20]), partial GLT-1 loss or A β PP/PS1 transgene expression decreased tyrosine phosphorylation of the insulin receptor.

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

IRS-1 is a critical adapter protein that is responsible for transmitting signals from the insulin receptor to intracellular pathways [46]. IRS-1 is a key target for negative feedback regulation of insulin signaling [47, 48], and there is evidence that Akt activation may be in part responsible for regulation of IRS-1 [49].

At 6 months (Fig. 3C, D), we found a statistically significant difference among the four groups in the ratio of phospho-IRS-1(Ser^{632/635})/IRS-1 ($F[3,18] = 3.885$, $p = 0.027$). Phospho-IRS-1/IRS-1 ratios were significantly elevated by GLT-1 heterozygosity, whether or not the animals expressed an A β PP/PS1 transgene (GLT-1: $F[1,18] = 10.183$, $p = 0.005$; A β PP: $F[1,18] = 0.296$, n.s.). While there appeared to be a similar trend of elevated IRS-1 Ser^{632/635} phosphorylation among the GLT-1_{het} animals (Fig. 3B, D), 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 A β PP/PS1 transgene status (GLT-1: $F[1,20] = 3.442$, n.s.; A β PP: $F[1,20] = 3.486$, 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 [49]. To address this possibility, we assessed mTOR activity by measuring phosphorylation at Ser²⁴⁴⁸. For further confirmation of any changes in mTOR activity, we also measured phosphorylation of p70 S6 kinase (p70 S6K-Thr³⁸⁹), a downstream target of mTOR often used as an indicator of mTOR signaling [50, 51]. We found no statistically significant changes in the ratios of phospho-mTOR (Ser²⁴⁴⁸) to total mTOR or phospho-p70 S6K (Thr³⁸⁹) to total p70 S6K at either 6 months (mTOR: means $\pm$ 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}/A β PP/PS1, and GLT_{het}/A β PP/PS1, respectively) or at 9 months of age (mTOR: means $\pm$ 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}/A β PP/PS1, and GLT_{het}/A β PP/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 IDE expression in AD, which also degrades A β [33, 37, 38, 52, 53]. In addition, decreased phosphorylation of IR β (Tyr^{1150/1151}) is also associated with reduced IDE activity in mice with impaired insulin signaling due to a high fat diet [36]. In order to address whether GLT-1 heterozygosity and/or A β PP/PS1 transgene expression alters

IDE activity in the brain, we measured the levels of 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 1 shows that partial loss of GLT-1, but not A β PP/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: $F[1,40] = 6.930$, $p = 0.012$; A β PP: $F[1,40] = 0.704$, n.s.; Age: $F[1,40] = 0.902$, n.s., respectively). Table 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.; A β PP: $F[1,38] = 0.370$, 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 [38, 52], even at early stages of the disease [53]. This also raises an interesting question as to why overall amyloid levels were not elevated in these mice [20] in that IDE degrades both A β_{40} and A β_{42} [54]. While there are multiple proteases capable of catabolizing A β [55], another robust means of clearing CNS A β involves A β transiting the blood-brain barrier into the peripheral circulation followed by rapid degradation in the liver [56–59]. Both IDE and GLT-1 are highly expressed in liver [60, 61]. In addition, the liver has been shown to regulate A β clearance from the brain by affecting circulating peripheral A β levels [59].

To determine whether partial GLT-1 loss influenced IDE activity in the liver, we measured 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 shows that partial GLT-1 loss, but not expression of the A β PP/PS1 transgene, caused a marked, significant increase in IDE activity in the liver (GLT-1: $F[1,36] = 42.717$, $p < 0.001$; A β PP: $F[1,36] = 3.457$, 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.; A β PP: $F[1,36] = 0.252$, n.s.), but were overall higher in the older mice (Age: $F[1,36] = 5.115$, $p = 0.030$).

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 A β clearance.

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 [62]. 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 [5].

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 [23]. Also, recent findings show that synaptic activity regulates co-localization of mitochondria with GLT-1 at sites of glutamate uptake [63]. Significantly, a number of studies show that treatments which activate the IR/IRS-1/Akt signaling cascade [32, 64] can regulate GLT-1 expression levels [25–27]. For example, Li and colleagues [27] 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. More importantly, both the *in vitro* [27] 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 [65], 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 [1, 66]. Moreover, GLT-1 expression levels in the pancreas are considered too low to be functionally significant in clearing extracellular pancreatic glutamate [66]. 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 [20].

Involvement of GLT-1 in AD-related pathology

At 6 months of age, but not at 9 months, the mice studied in this report exhibited a significant increase in the ratio of A β ₄₂/A β ₄₀ among GLT-1 heterozygotes compared to animals with normal, wild-type GLT-1 levels [20]. While this increase was modest, this raises the possibility that A β ₄₂ levels might have contributed to the overall pattern of altered Akt, IR β , and IRS-1 phosphorylation, which collectively was more pronounced in the 6-month-old animals than in the 9-month-old mice. Other investigators have reported that exposing primary cultured neurons to oligomeric A β ₄₂ (also known as amyloid- β -derived diffusible ligands, or ADDLs) increases phospho-Akt (Ser⁴⁷³) with an accompanying reduction in phospho-IR β (Tyr^{1150/1151}) levels [35]. In addition, glutamate exposure (simulating synaptic glutamate release) and treatments that stimulate neuronal glutamate release also reduced phospho-IR β (Tyr^{1150/1151}) [35]. These data are consistent with our

findings which show that both partial GLT-1 loss and expression of an A β PP^{swe}/PS1 E9 transgene increases Akt (Ser⁴⁷³) phosphorylation; and is associated with a similar reduction in IR β (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 [16–19]. Such losses would not be expected to prevent restoration of basal extracellular glutamate levels following synaptic glutamate release [22, 67]. 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 [68, 69]. In keeping with this, we have recently reported that exposing hippocampal slices to oligomeric A β ₄₂ reduces GLT-1 activity, thereby approximately doubling the time required to clear endogenous, synaptically released glutamate by specifically reducing GLT-1 activity [21].

In this report, we found that partial GLT-1 loss, but not A β PP/PS1 mutant transgene expression, reduced IDE activity levels in the brain (Table 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 [33, 37, 38, 52, 53, 70, 71]. Whether GLT-1-specific reductions in IDE activity are driven by changes in Akt, IR β , 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-1 in zone 3 hepatocytes [65, 72]. While we found a significant increase in the ratio of A β ₄₂/A β ₄₀ in these mice at 6 months of age, we did not find any other significant changes in A β accumulation between the GLT_{wt}/A β PP/PS1 and GLT_{het}/A β PP/PS1 groups at either 6 or 9 months [20]. CNS A β is efficiently cleared into the peripheral circulatory system, which is often referred to as a peripheral A β sink [56, 73]. We and others have shown that the liver functions as a critical systemic A β drain [58, 59, 74, 75]. Importantly, peripheral A β clearance by the liver can regulate CNS A β clearance [59]. 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 A β 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, IR β , 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 [20], thereby strengthening the idea that GLT-1 loss can drive cognitive impairments via mechanisms that involve early-occurring insulin/Akt signaling defects.

Overall, these findings are in keeping with the idea that GLT-1 loss primarily influences AD-related neuropathology early on in the pathogenic process, prior to later time points when

amyloid deposition becomes a more dominant pathological factor in these A β PP/PS1 transgenic mice that accumulate A β at higher rates after 6 months [39].

These data linking GLT-1 loss to insulin/Akt 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 [16] 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 heterozygous mice express 50–60% of the GLT-1 wild-type levels from birth. It is possible this could contribute to complex interactions between GLT-1 heterozygosity and prolonged A β PP/PS1 transgene expression that could also influence the prominence of GLT-1 loss on cognition and Akt/insulin signaling at earlier, rather than later time points.

Summary

In conclusion, the findings in this report demonstrate for the first time that partial GLT-1 loss causes sustained Akt activation *in vivo* that is associated with feedback-related impairment of IR/IRS-1 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 disturbances in Akt and the IR/IRS-1 pathway that occur in AD [32–34].

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Fig. 1.

Akt phosphorylation is increased by partial loss of GLT-1 and expression of the AβPP/PS1 transgene and is more pronounced at 6 months of age. A, B) Western blots from 6 and 9 month animals, respectively. C, D) Indicating sustained activation of Akt, densitometric quantification of blots in (A) and (B) revealed a statistically significant increase in the ratio of phosphorylated Akt (Ser⁴⁷³) to total Akt in GLT_{het} and AβPP/PS1 transgene mice compared to GLT-1_{wt}/nTg mice at both 6 (C) and 9 (D) months of age ($p = 0.01$ and $p = 0.001$, respectively). Each lane represents one animal. Data are presented as normalized means $\pm$ SEM ($n = 5-6$ mice per group).

Fig. 2.

Insulin receptor autophosphorylation is reduced by partial loss of GLT-1 and AβPP/PS1 at 6 months of age. A, B) Western blots from 6 and 9 month animals, respectively. C, D) Indicating reduced insulin receptor activation, densitometric quantification of blots in (A) and (B) revealed a statistically significant decrease ($p = 0.039$) in the ratio of Tyr^{1150/1151} phosphorylated IRβ to total IRβ in GLT_{het} and AβPP/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 $\pm$ SEM ($n = 6$ mice per group).

Fig. 3.

IRS-1 serine phosphorylation is increased by partial loss of GLT-1 at 6 months of age. A, B) Western blots from 6 and 9 month animals, respectively. C, D) Indicating reduced IRS-1 signaling, densitometric quantification of blots in (A) and (B) revealed a statistically significant increase ($p = 0.027$) in the ratio of Ser^{632/635} phosphorylated IRS-1 to total IRS-1 in mice with partial loss of GLT-1, but not animals with an A β PP/PS1 transgene at 6 months of age (C). Phospho-IRS-1/IRS (Ser^{632/635}) ratios were not significantly different in 9-month-old mice (D). Each lane represents one animal. Data are presented as normalized mean $\pm$ SEM ($n = 5-6$ mice per group).

Table 1

In brain, partial loss of GLT-1 reduced 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	1.00 ± 0.04	1.00 ± 0.08
GLT _{wt} /AβPP/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} /AβPP/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 [¹²⁵I]-insulin and were normalized to samples incubated with excess non-radiolabeled insulin. Data were 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^{+/-} versus GLT-1^{+/+}) loss caused a significant decrease in IDE activity ($p < 0.012$), while the effects of AβPP/PS1 transgene expression (AβPP/PS1 versus non-transgenic) and age (6 versus 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 significant differences for any factors (GLT-1 genotype, AβPP/PS1 expression, or age). Data are presented as mean ± standard error of the mean (SEM). $n = 5-6$ mice per group.

Table 2
In liver, partial loss of GLT-1 increased 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	1.00 ± 0.10	1.00 ± 0.03
GLT _{wt} /AβPP/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} /AβPP/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 [¹²⁵I]-insulin and were normalized to samples incubated with excess non-radiolabeled insulin. Data were 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^{+/-} versus GLT-1^{-/-}) loss caused a significant increase in IDE activity ($p < 0.001$), while the effects of AβPP/PS1 transgene expression (AβPP/PS1 versus non-transgenic) and age (6 versus 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, AβPP/PS1 expression, and age) revealed a statistically significant difference between the two ages. Upon further analysis, a two-way ANOVA (GLT-1 genotype and AβPP/PS1 expression) of 6 month data revealed no significant differences, while at 9 months there was only a significant effect of AβPP/PS1 expression ($p < 0.019$). Data are presented as mean±standard error of the mean (SEM). $n = 2-9$ mice per group.