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Effects of ß-amyloid on glucose uptake by cultured hippocampal neurons

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ABSTRACT

Alzheimer's disease (AD) is the most common type of dementia in the elderly. The deposit of extracellular amyloid ß–peptide (Aß) is a distinct feature of the disease. Recent studies have shown that Aß interferes with glucose uptake in cultured neurons; however, how Aß inhibits glucose uptake is not know. This study proposed a pathway in which Aß25-35, a neurotoxic portion of the Aß peptide identical to the 25–35 amino acid-sequence in Aß, decreases neuronal glucose uptake. The inhibitory effect of Aß25-35 on neuronal glucose uptake was reduced by a G protein antagonist (GPAnt-2). In the first part of the study, we demonstrated a signaling pathway in which Aß25-35, a neurotoxic portion of the Aß peptide corresponding to amino acids 25-35, inhibits neuronal glucose uptake by hippocampal neurons. The GPAnt-2, which blocks Gs, prevented the inhibitory effect of Aß on the glucose uptake. Cholera toxin, which stimulates adenylyl cyclase by activating Gs protein, also inhibited neuronal glucose uptake. Furthermore, the inhibitory effect of cholera toxin on glucose uptake was potentiated by Aß. Exposure of cells to Aß resulted in a transitory increase in intracellular levels of cAMP. Addition of dibutyryl cAMP (Bt_2cAMP) or an adenylyl cyclase activator, forskolin, to the culture medium inhibited neuronal glucose uptake, and a protein kinase A inhibitor (KT-5720) blocked the Aß-mediated inhibition of glucose uptake. Thus, our findings suggest that Aß inhibits glucose uptake by activating the Gs-coupled receptors and involves the cAMP-PKA system. The second part of this study examined the effects of insulin and insulin-like growth factor 1 (IGF-I) on the inhibitory effect of Aß on neuronal glucose uptake. Insulin and IGF-I elevated neuronal glucose uptake, but the effect of IGF-I was more potent than insulin. Neurons exposed to Aß25-35 showed 38% less glucose uptake than the control. However, IGF-I and insulin prevented this inhibitory effect of Aß. To study the signaling pathway of IGF-I that stimulates glucose uptake, hippocampal neurons were studied for their glucose uptake following exposure to a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002) or a mitogen-activated protein kinase (MAPK) inhibitor (PD-98059). The LY294002 decreases the stimulatory effect of IGF-I on neuronal glucose uptake while PD-98059 has no effect on the IGF-I

increase neuronal glucose uptake. The results demonstrates that (1) both IGF-I and insulin are effective in elevating neuronal glucose uptake and preventing the inhibitory effect of Aß and (2) PI3K plays a regulatory role in neuronal glucose uptake. The present study shows that Aß may inhibit neuronal glucose uptake via G protein and cAMP-dependent protein kinase pathway and insulin and IGF-I reverse the Aß inhibition effect on neuronal glucose uptake. However, how the two hormones interact to the Aß inhibition effect need to be further investigated.

CHAPTER 1. GENERAL INTRODUCTION

Neurons in Alzheimer's disease

Alzheimer's disease (AD) claims 65% of the dementias found in the elderly [70]. AD is characterized by deposition of amyloid ß-peptide (Aß) in many areas of the brain. This extra-cellular peptide lies in the neuropathological lesion, the neuritic plaque. Neuritic plaques are classified into two types: a diffuse amyloid plaque which consists only of an amorphous form Aß and a mature plaque, which consists of a core of aggregated amyloid filament surrounded by dystrophic neurites and reactive glial cells [87]. Aß is derived from a transmembrane glyco-protein called Aß precursor protein (ßAPP) [45]. It is a 39 - 43 amino acid peptide cleaved from extracellular and transmembrane domains of ßAPP [86].

The evidence supporting Aß deposition as the primary pathological process is the iden-tification of pathogenic mutations in the Aß precursor protein gene and neuropathology similar to AD in transgenic mice overexpressing ßAPP [27,42]. Although degenerating neurons in transgenic mice accompany the Aß deposition, the mechanisms involved in Aß-mediated neu-ronal degeneration are not well understood, but it has been shown that the neurotoxic portion of Aß is amino acids 25-35 (Aß25-35) of the full-length Aß peptide [62,113]. In primary cell cultures, Aß is neurotoxic [73,88,114]. This toxic effect seems to depend on the stage of neu-ronal differentiation and the aggregation stage of Aß [73,74,92]. Thus, Aß was neurotoxic only to mature hippocampal neurons [114]. But even neurons at the early stages undergo neurodegeneration if exposed to aggregated Aß [73]. Although synthetic Aß has been shown to have neuronal toxicity on cultured cells, injecting this synthetic peptide into the brain of young rats did not induce neuronal degeneration. In contrast, injection of Aß (Aß1-40) into old rats did result in neurodegeneration [52]. Similarly, in the brain of the aging rhesus monkey, Aß caused neurodegeneration, while it did not in the brain of the young rhesus monkey [28], suggesting that Aß-induced neuronal death is specific to aging.

Aß-mediated inhibition of neuronal glucose uptake

In vitro study has shown that Aß impairs glucose uptake in hippocampal neurons [60] and astrocytes [69]; however, it is not known whether Aß affects neuronal and astrocytic glucose uptake in AD patients. There is some evidence suggesting that glucose metabolism is changed in the brains of AD patients [20.46.76.78]. Studies by positron emission tomography (PET) scans detected a decrease in glucose uptake in the cerebral cortex [20.46.76.78]. It was calculated that reduction of cerebral glucose utilization ranges from 19% in mild cases to 40% and more in severe cases of AD [41.54]. Immunohistochemical study further revealed a decrease in glucose transporters (GLUT1. GLUT3) in several brain regions, including the hippocampus. the area commonly affected in AD patients [36.93]. Reduced glucose uptake by individuals genetically at risk for developing AD also supports the notion that glucose deprivation precedes neuronal degeneration [49.72.77]. Although *in vitro* effect of Aß may not accurately represent an *in situ* situation. previous *in vitro* experiments [2.61] have often proved their usefulness in simulating cellular responses *in situ* [29.30.32]. Thus, it is likely that the decreased cerebral glucose uptake in AD may reflect. in part, the inhibitory effect of Aß on neuronal and astrocytic glucose uptake.

In healthy, nonstarved mammalian brain. glucose is the only substrate for the formation of energy in the form of ATP [21]. Glucose deprivation results in increased use of endogenous substrates, depletion of ATP, membrane depolarization, extracellular accumulation of excitatory amino acids, loss of neuronal homeostasis and ultimately neuronal cell death [61]. Neurons interfered with glucose uptake or exposed to Aß result in impaired mitochondrial activity and suppressed production of ATP [61]. Based on the rates of oxidized glucose and oxygen, it was estimated that the rate of cerebral ATP formation decreases by 7% in early-onset AD, and 20% in late-onset AD [39]. Such metabolic impairment of neurons was shown to increase their vulnerability to glutamate toxicity and oxidative insults [61]. It was shown that Aß inhibits astrocytic glutamate uptake [69] and stimulates microglial release of toxic levels of nitric oxide [8].

Thus, their functional changes by Aß may be fatal to those neurons suffering from glucose deficiency.

Regulation of neuronal glucose

The mechanism involved in Aß inhibition of glucose uptake is not known. It was suggested that lipid peroxidation by Aß is involved in impairment of neuronal glucose transport [60]. Lipid peroxidation generates 4-hydroxynonenal (HNE). Since HNE binds to neuronal glucose transporter GLUT3 and exogenous application of HNE induces an impairment of glucose transport, it is suggested that HNE binding with GLUT3 inhibits glucose transport in neurons. However, there is no direct evidence at present showing functional impairment of GLUT3 by HNE binding. In spite of importance of glucose for neural functions, very little is known about the regulatory mechanisms involved in neuronal glucose uptake, GLUT3 expression, and GLUT translocation from cytoplasmic pool to plasma membrane. In neurons, the rate of glucose uptake depends on the number of GLUT3 on the plasma membrane, not on extracellular glucose concentration [71]. In cultured hippocampal neurons, Aß did not affect GLUT3 protein levels, even after 6 hr of Aß treatment [60]. This suggests that Aß induces an intrinsic functional change in GLUT3 on the plasma membrane. In adipocytes, phosphorylation of GLUT4 is known to inhibit insulin-stimulated glucose transport [47,57]. Phosphorylation of GLUT4 is confined to the intracellular domain at the COOH-terminal region and this terminal region appears to play an important role in the regulation of transport activity [73]. In Chinese hamster ovary (CHO) cells, changing Ser[488] to an Ala prevented phosphorylation of GLUT4: however, dibutyryl cAMP (Bt$_2$cAMP) was still able to inhibit glucose transport even after this mutation [75]. Although the data seems to suggest that phosphorylation is not required for the inhibition of GLUT4, it is not conclusive. The effect of phosphorylation may also depend on the cell type in which the transporter is expressed. For example, the function of GLUT4 is to transport glucose in response to insulin, a function not present in CHO cells [43,91]. It was also shown that,

in CHO cells, cAMP-dependent PKA had no effect on glucose uptake [75]. Interestingly, PKA inhibitor, H-89, was shown to prevent Aß-induced apoptotic cell death [99]. It is, therefore, conceivable that phosphorylation of GLUT3 inhibits neuronal glucose uptake. Aß inhibition of neuronal glucose uptake could also be mediated by Ca^{2+}/calmodulin-sensitive adenylyl cyclase and increase cAMP levels, the process known to occur in the hippocampus [65]. However, Ca^{2+}/calmodulin-mediated inhibition of glucose uptake is less likely because it was shown that Aß-induced apoptosis was not inhibited by KN-62, an inhibitor of Ca^{2+}/calmodulin dependent protein kinase [98].

To date the role of cAMP in the regulation of neuronal glucose is not known. Forskolin was shown to stimulate adenylate cyclase, elevating the intracellular cAMP level and inhibiting the glucose uptake by neurons and astrocytes [34]. Dibutyryl cAMP was also shown to inhibit glucose uptake in L6 myoblast [58] and hamster ovary cells [73]. In vitro study suggests that Aß inhibits glucose uptake by activating the cAMP-PKA system. How these *in vitro* data relate to the decreased glucose metabolism in AD is not certain. It is possible that the decreased cerebral glucose uptake shown in AD may reflect, in part, the inhibitory effect of Aß on neuronal and astrocytic glucose uptake. Such an hypothesis infers the presence of functional G protein-stimulated activity of adenylyl cyclase and cAMP in AD patients. Postmortem studies of AD patients have shown normal G-protein levels [12,63,79,104], catalytic activity of cAMP, and PKA [7,24,64,81]. However, the regulatory function of G protein on adenylyl cyclase was reported as either normal [79] or reduced [12,24,84]. This inconsistency may reflect differences in postmortem instability of the enzyme at different stages of Alzheimer's disease [7]. The regulatory function of G protein on adenylyl cyclase could also be affected only at the terminal stage of AD disease.

Protective role of insulin and insulin-like growth factor (IGF) on Aß inhibition of neu-ronal glucose uptake

Neurons exposed to Aß become more susceptible to glucose deprivation [11]; but. IGF-I and IGF analogues are effective in promoting cell survival under such condition [10,35]. Furthermore. IGF-I is shown to protect. and more importantly, to rescue rat hippocampal neu-rons against Aß toxicity [18]. The protective mechanism of IGF-I is not known, but it was shown that insulin and IGF-I act as a neuronal growth factor [89,103]. In transgenic mice that overexpress IGF-I in the brain. glucose uptake increases significantly in the brain [31]. *In vitro* studies have also shown that IGF-I increases glucose uptake in skeletal muscle. L6 myotubes and PC12 cells [23,37,56,111], while insulin increases glucose uptake in PC 12 cells [111]. Although several studies in humans suggest the importance of insulin in hippocampal energy metabolism [26,40] and memory facilitation [13,14], it is not known whether insulin and IGF-I promote glucose uptake by neurons. In both skeletal muscle and adipocytes. insulin and IGF-I were shown to stimulate translocation of GLUT4. increasing the number of GLUT4 in the plasma membrane [59,71,110]. Similarly, insulin and IGF-I were shown to translocate GLUT3 in L6 myotubes [67]; however. what regulates translocation of neuronal GLUT3 is not known at present.

Whether insulin can regulate glucose metabolism within the brain has been a contro-versy for some time. An increasing body of evidence suggests that insulin has a vital role in brain function [108]. Expression of the insulin gene and insulin synthesis occur in a highly specific pattern, with the highest density in hippocampal pyramidal neurons [17]. Plasma insu-lin was also shown to cross the blood-brain barrier by saturable receptor-mediated transcytosis. and residual insulin is removed through the CSF [3,5,85]. At present. normal insulin and IGF-I levels in the human brain are not known. However, a deficit in these hormones is suspected in the brains of AD patients because of their higher plasma insulin and IGF-I, and lower CSF

insulin levels [15]. Insulin and IGF-I receptors are also present in the human brain [68,80,83], and their numbers increase in the brains of AD patients [6,16,25]. If this proliferation of insulin and IGF-I receptors is the brain's attempt to compensate for a lack of insulin and IGF-I, the reduced cerebral glucose utilization found in AD may reflect the cerebral insulin and IGF-I deficit in AD [41]. IGF is synthesized primarily in the liver, but it is also made by neurons in the developing brain [4].

Alzheimer's patients have abnormally low blood glucose [44] and infusion of glucose has been shown to enhance memory in Alzheimer's patients and normal elderly adults [13,50]. Because glucose infusion is usually associated with elevation of plasma insulin, a group of Alzheimer's patients was further assessed by raising plasma insulin, but preventing a rise in plasma glucose [14]. There was a striking memory enhancement in these patients, suggesting a functional importance of insulin in the brain. Insulin levels in CSF can be elevated by oral glucose load in patients with AD [26]. Furthermore, intraventricular administration of insulin exerts anabolic effects on hippocampal energy metabolism. In contrast, intraventricular injection of streptozotocin (STZ), the inhibitor of tyrosine kinase of the insulin receptor, caused significant decreases in glucose utilization and ATP levels [40]. At present, insulin levels in the human brain is not known; however, insulin deficit in the brains of Alzheimer's patients is suspected because of their higher plasma insulin and lower CSF insulin levels in Alzheimer's patients [15]. The proliferation of insulin and IGF-I receptors in AD [16,25] was interpreted as the brain's attempt to compensate for a lack of insulin and IGF-I. The insulin deficit in the brain may also be responsible for the reduced cerebral glucose utilization found in AD [41].

Insulin and IGF-I share structural and functional features, including an α-subunit that binds the hormone-like agent and a ß-subunit that has a tyrosine-specific protein kinase [101]. The metabolic effects of these hormones are initiated by their binding to the extracellular domain of the specific membrane receptors, which lead to the activation of, among others, Ras and phosphoinositide 3-kinase (PI3K) [33,90]. Many of these kinases appear to be arranged in

cascades, including a cascade that results in the elevation of glucose uptake in insulin sensitive cells [100]. The signaling pathways that stimulate neuronal glucose uptake are not known. In adipocytes, PI3K was shown to increase GLUT4 translocation to the plasma membrane and glucose transport [22,38,48,51]. Furthermore, PI3K inhibitor, LY294002, was shown to inhibit glucose uptake [82] by interfering with insulin mediated translocation of GLUT4 [9].

The short-term metabolic effect of insulin (e.g., increase in glucose transport) appears to require PI3K. In both skeletal muscle and adipocytes, insulin and IGF-I control the actual number of GLUT4 in the plasma membrane by stimulating translocation of GLUT4 from an intracellular store to the plasma membrane [59,71,110]. Similarly, insulin and IGF-I were shown to translocate GLUT3 in L6 myotubes [67]; however, what regulates translocation of neuronal GLUT3 is not known at present. In adipocytes, PI3K mediates the following events: (i) insulin-induced reorganization of actin filaments [106], (ii) activation of 3-phosphoinositide-dependent PKB [1] and PKC-zeta [94,95], (iii) GLUT4 translocation to the plasma membrane, (iv) glucose transporter exocytosis [112] and (v) glucose transport [22,38,48,51]. Very little is known about PKB and PKC-zeta in their role in glucose metabolism. Activation of PKB induces increase in glucose uptake, glycogen synthesis, and protein synthesis in L6 myotubes. Such PKB action is independent of Ras and MAPK activation [19,55,100,102]. Insulin also activates PKC-zeta through PI3K, contributing to the activation of GLUT4 translocation [94,95]. Thus, PKB and PKC-zeta appear to act as key enzymes for multiple biological functions of insulin, including glucose uptake. PKB may also stimulate glucose uptake by phosphorylating GLUT3, much like PKB in adipocytes that phosphorylates phosphodiesterase 3B (PDE-3B) [109]. The MAPK cascade, on the other hand, may have a role in the regulation of gene transcription, and hence the more long-term effect of insulin [66]. Insulin and IGF-I activate RasGTP and MAPK cascade in 3T3-L1 adipocytes, skeletal muscle and myotubes [19,53,55,96,102,107]. Chronic (16 hr) treatment with insulin was shown to increase GLUT3 mRNA and protein in UMR 106-01, a clonal osteosarcoma cell line [97], while, IGF-I induced p53 mRNA in cardiac muscle cells

[105]. If insulin deficit occurs in the brain and such deficit accompanies deposition of Aß, it is conceivable that the long-term neuronal effect of such condition include significant reduction in numbers of GLUT3, and subsequent decrease in glucose uptake.

Although there are numerous published reports linking Aß to neurotoxicity *in vitro* and *in vivo*, research investigating the mechanisms involved in reduced glucose utilization in AD has been primarily focused on free radicals generated by Aß [61]. It has been shown by others that neurons exposed to Aß become more susceptible to glucose deprivation; but, IGF-I and IGF analogues are effective in promoting cell survival under such condition. Furthermore, IGF-I is shown to protect and rescue rat hippocampal neurons against Aß toxicity. Studies in human also suggest the importance of insulin in hippocampal energy metabolism [26,40] and memory facilitation [13,14]. Reduced cerebral glucose uptake by individuals genetically at risk for developing AD also supports the notion that glucose deprivation precedes neuronal degeneration [49,72,77]. A perturbed control of glucose breakdown may severely damage cellular homeostasis and subsequently causes not only a substantial impact on cellular function, but also increased neuronal vulnerability to, among others, glutamate toxicity and oxidative insults. Therefore, understanding the mechanisms involved in neuronal glucose regulation by Aß and hormones (insulin and IGF-I) are a critical importance for improving neuronal glucose utilization in AD.

Research objectives

There were two research objectives. The first objective was to test the hypothesis that G protein and cAMP mediate Aß25-35 inhibition of neuronal glucose uptake. This part was carried out by studying the effect of (1) G protein antagonist on glucose uptake, (2) cholera toxin on glucose uptake, (3) Aß25-35 on intracellular cAMP, (4) cAMP on glucose uptake, and (5) a protein kinase A (PKA) inhibitor (KT-5720) on glucose uptake. The second objective was to test the hypothesis that insulin and IGF-1 prevent the inhibitory effect of Aß on neuronal glu-

cose uptake by either PI3K or MAPK. This part was carried out by studying the effect of Aβ25-35 on neuronal glucose uptake under the influence of (1) insulin or IGF-I and (2) PI3K inhibitor (LY294002) or mitogen-activated protein kinase (MAPK) inhibitor (PD-98059).

Dissertation organization

This dissertation is organized into 4 chapters: a general introduction, two papers prepared for submission, and a general discussion. Each chapter has individual references and figures at the end. The papers are being submitted for publication to *Nature* and *Brain Research*, respectively. The general discussion addresses the finding of both papers.

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CHAPTER 2: G PROTEIN AND CAMP-DEPENDENT PROTEIN KINASE (PKA) MEDIATE AMYLOID ß-PEPTIDE INHIBITION OF NEURONAL GLUCOSE UPTAKE

A paper to be submitted to *Nature*

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Alzheimer's disease (AD) is characterized by deposition of amyloid ß-peptide (Aß), a 39-43 amino acid protein[1]. Aß has been shown to impair glucose uptake in cultured hippocampal neurons[2] and astrocytes[3]. It is not known whether Aß affects neuronal glucose uptake in the brains of AD patients; however, ample evidence exists to suggest that glucose metabolism has changed in their brains[4-11]. The mechanism by which Aß inhibits glucose uptake in cultured cells is not known. Here we demonstrated a signaling pathway in which Aß25-35, a neurotoxic portion of the Aß peptide corresponding to amino acids 25-35[12], inhibits neuronal glucose uptake by hippocampal neurons. The GP antagonist-2[13], which blocks Gs, prevented the inhibitory effect of Aß on the glucose uptake. Cholera toxin, which stimulates adenylyl cyclase by activating Gs protein, also inhibited neuronal glucose uptake. Furthermore, the inhibitory effect of cholera toxin on glucose uptake was potentiated by Aß. Exposure of cells to Aß resulted in a transitory increase in intracellular levels of cAMP. Addition of dibutyryl cAMP (Bt_2cAMP) or an adenylyl cyclase activator, forskolin, to the culture medium inhibited neuronal glucose uptake, and PKA inhibitor KT-5720 blocked the Aß-mediated inhibition of glucose uptake. Thus, our findings suggest that Aß inhibits glucose uptake by activating the Gs-coupled receptors and involves the cAMP-PKA system.

To examine whether Aß inhibition of neuronal glucose uptake is mediated by a G protein, hippocampal neurons were exposed to GP antagonist-2, which blocks the receptor-Gs interaction[13]. In this study, cultured hippocampal neurons at day 4 post-plating were exposed to synthetic Aß25-35 for 24 hr with or without 10 µM GP antagonist-2 in the culture medium.

The neuronal glucose uptake was measured and expressed as picomol [^{14}C] D-glucose per mg of cellular protein[3]. Aß25-35 significantly inhibited neuronal glucose uptake [$F_{(3,8)}=66.8$, $p=0.0001$] (Fig. 1a). Those neurons exposed to Aß25-35 showed 40% less glucose uptake compared with the control. However, the G protein antagonist prevented this inhibitory effect of Aß, suggesting G protein mediates Aß inhibition of neuronal glucose uptake.

To further assess the role of Gs on the inhibitory effect of Aß on neuronal glucose uptake, the effect of cholera toxin, which stimulates adenylyl cyclase by activating Gs[14], was studied in cultured hippocampal neurons. In this study, neuronal glucose uptake was assessed at day 4 post-plating following exposure to cholera toxin (0.01, 0.1 or 1 µg/ml) in the medium for one hr. Cholera toxin at 0.01 and 0.1 µg/ml had no effect on neuronal glucose uptake, but 1 µg/ml cholera toxin significantly inhibited glucose uptake [$F_{(3,8)}=38.63$, $p=0.001$] (Fig. 1b). Based on this observation, the two low doses of cholera toxin (0.01, 0.1 µg/ml) were used to study whether this toxin potentiates the inhibitory effect of Aß. In this study, neurons at day 4 post-plating were exposed for 24 hr to both Aß25-35 coated on cover slips and cholera toxin at either 0.01 or 0.1 µg/ml in the culture medium. Those neurons exposed to Aß25-35 showed 38% less glucose uptake than the control ($t=3.78$, $p=0.0001$). When neurons were exposed to both Aß25-35 and 0.1 µg/ml cholera toxin, their glucose uptake was 53% less than the control ($t=5.99$, $p=0.001$) and 26% less than those exposed to Aß25-35 alone ($t=4.87$, $p=0.0004$). Thus, cholera toxin potentiated the effect of Aß25-35, suggesting that Aß inhibits glucose uptake by activating the Gs-coupled receptors.

Since Gs activates adenylyl cyclase[15], we studied the effect of Aß on intracellular cAMP levels in cultured hippocampal neurons. In this study, neurons at day 4 post-plating were exposed to Aß25-35 for 2 hr, 2 days, or 3 days. Neurons were analyzed for cAMP content using radioimmunoassay (Amersham). A significant change in intracellular cAMP levels was observed in neurons exposed to Aß [$F_{(1,12)}=75.5$, $p=0.0001$] and this change was time-dependent [$F_{(2,12)}=19.3$, $p=0.0002$] (Fig. 2a). The highest cAMP level was found in neurons ex-

posed to Aß for 2 hr. followed by those neurons exposed to Aß for 48 hr. cAMP levels at 72 hr post-exposure were not different from the control.

An elevation of intracellular cAMP levels by Aß25-35 within 2 hr of application suggests that Aß stimulates Gs-adenylyl cyclase coupling. To further assess the role of cAMP in neuronal glucose uptake. we studied the effect of cAMP on neuronal glucose uptake in hippocampal neurons. At post-plating day 4. cultured neurons were exposed for one hr to either Bt_2cAMP (0, 0.5. 1. 5. 10. 50 μM) or forskolin (0. 10. 25. 50. 100 μM). Neurons were assessed for glucose uptake and cellular protein at 0 and 1 hr post-treatment. Neuronal glucose uptake was significantly inhibited by Bt_2cAMP [$F(5. 18)=9.07$, $p=0.0002$] (Fig. 2b) or forskolin [$F(4. 15)=4.68$. $p=0.0006$] (Fig. 2c). This inhibitory effect was dose-dependent for both Bt_2cAMP ($r^2=0.54$) and forskolin ($r^2=0.58$). Neurons exposed to either 50 μM Bt_2cAMP or 100 μM forskolin showed only 13% of glucose uptake by the control neurons. Since Aß elevated intracellular cAMP levels within 2 hr. we assessed the inhibitory effect of Aß on glucose uptake following exposure for 3 hr to Aß25-35. Glucose uptake was significantly inhibited by Aß25-35 ($t=2.97$. $p=0.025$) (Fig. 2d), suggesting an acute inhibitory effect of Aß25-35.

Inhibition of neuronal glucose uptake by Bt_2cAMP and forskolin suggests that glucose uptake is regulated by cAMP-dependent protein kinase (PKA). To examine whether PKA plays a regulatory role in neuronal glucose uptake. the effect of a PKA inhibitor KT-5720 was studied in cultured hippocampal neurons. At day 4 post-plating, neurons were exposed to KT-5720 (0.1 μM and 1 μM) in the culture medium and Aß25-35 on cover slips for 24 hr. Neuronal glucose uptake and cellular protein were assessed at 1 hr post-sampling time. Glucose uptake by control neurons was 40 pmol/mg cellular protein (Fig. 3). In contrast. Aß25-35 significantly reduced glucose uptake to 15 pmol/mg cellular protein. i.e.. 38% of the control neurons. This inhibitory effect of Aß25-35 was prevented by 1 μM KT-5720. but not by 0.1 μM KT-5720. This dose-dependent effect of the PKA inhibitor was highly significant [$F(3. 16)=8.62$, $p=0012$]. The result suggests an inhibitory role by PKA on neuronal glucose uptake.

Discussion

The present study demonstrates that G protein and cAMP mediate Aß25-35 inhibition of neuronal glucose uptake. How these *in vitro* data relate to the decreased glucose metabolism in AD is not certain. It is possible that the decreased cerebral glucose uptake shown in AD may reflect, in part, the inhibitory effect of Aß on neuronal and astrocytic glucose uptake. Such an hypothesis infers the presence of functional G protein-stimulated activity of adenylyl cyclase and cAMP in AD patients. Postmortem studies of AD patients have shown normal G-protein levels[14,16-18], catalytic activity of cAMP, and PKA[19-22]. However, the regulatory function of G-protein on adenylyl cyclase was reported as either normal[14] or reduced[16,20,23]. This inconsistency may reflect differences in postmortem instability of the enzyme at different stages of Alzheimer's disease[19]. The regulatory function of G-protein on adenylyl cyclase could also be affected only at the terminal stage of AD disease. In this respect, our study seems to suggest that chronic exposure to Aß desensitizes Gs in regulating adenylyl cyclase. Aß inhibition of neuronal glucose uptake could also be mediated by Ca^{2+}/calmodulin-sensitive adenylyl cyclase and an increase cAMP levels, the process known to occur in the hippocampus[24]. However, Ca^{2+}/calmodulin-mediated inhibition of glucose uptake is less likely because it was shown that Aß-induced apoptosis was not inhibited by KN-62, an inhibitor of Ca^{2+}/calmodulin dependent protein kinase[25]. In neurons, the rate of glucose uptake depends on the number of GLUT3 on the plasma membrane, not on extracellular glucose concentration[26]. In cultured hippocampal neurons, Aß did not affect GLUT3 protein levels, even after 6 hr of Aß treatment[2]. This suggests that Aß induces an intrinsic functional change in GLUT3 on the plasma membrane. In adipocytes, phosphorylation of GLUT4 is known to inhibit insulin-stimulated glucose transport[27,28]. In our study, the PKA inhibitor, KT-5720, prevented the inhibitory effect of Aß on neuronal glucose uptake, suggesting GLUT3 phosphorylation by PKA as an important regulator of GLUT3 function in hippocampal neurons. Also consistent with our present findings, the

PKA inhibitor, H-89, was shown to prevent Aß-induced apoptosis[29]. Considering the importance of glucose in the CNS function, the effect of Aß on neuronal glucose metabolism in AD warrents further study.

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Acknowledgements

Figure legends

Figure 1. G protein mediates Aß inhibition of neuronal glucose uptake. Abbreviations used for figure: C=control, Aß=amyloid ß-peptide, Ga=GP antagonist 2, Ctx1=1 μg/ml cholera toxin, Ctx.1=0.1 μg/ml cholera toxin, Ctx.01=0.01 μg/ml cholera toxin. *a*, Cultured hippocampal neu-

rons at day 4 post-plating were exposed to synthetic Aß25-35 for 24 hr with or without 10 μM GP antagonist-2 in the culture medium. Those neurons exposed to Aß25-35 showed 40% less glucose uptake compared with the control. However. GP antagonist 2 prevented this inhibitory effect of Aß. *b*, Neuronal glucose uptake was assessed at day 4 post-plating following exposure to cholera toxin (0.01, 0.1 or 1 μg/ml) in the medium for one hr. Cholera toxin at 0.01 and 0.1 μg/ml had no effect on neuronal glucose uptake, but 1 μg/ml cholera toxin significantly inhibited glucose uptake. c, Two low doses of cholera toxin (0.01, 0.1 μg/ml) were used to study whether cholera toxin potentiates the inhibitory effect of Aß in neuronal glucose uptake. Neurons at day 4 post-plating were exposed to synthetic Aß25-35 for 24 hr with cholera toxin at either 0.01 or 0.1 μg/ml in the culture medium. As noted above (a), neurons exposed to Aß25-35 showed 38% less glucose uptake than the control. Neurons exposed to both Aß25-35 and 0.01 μg/ml cholera toxin did not differ in their glucose uptake from those exposed to Aß25-35 alone. However. those neurons exposed to both Aß25-35 and 0.1 μg/ml cholera toxin showed 26% less glucose uptake than those exposed to Aß25-35 alone.

METHODS. Aß25-35 and scrambled control peptide were prepared according to Parpura-Gill et al.[3]. Hippocampal neurons from 17E Sprague-Dawley rats were cultured according to Abe and Kimura[30]; however. we used N2 instead of N1 supplement in our cultures. Neurons plated onto 24-well tissue culture plates were exposed to Aß25-35 or scrambled control peptide by using cover slips coated with the peptides. The coated-side was placed on top of the cultured neurons at post-plating day 4. Neuronal uptake of glucose was assayed by using [^{14}C]D-glucose (313 μCi/mmol, ICN) at final glucose concentrations of 600 nM (0.1 μCi/500μl media in well). Cell lysates were used for determination of radioactivity by using a beta liquid scintillation counter (Packard, 1600TR) and for assay of total cell protein (Bio-Rad protein assay kit). Neuronal uptake of [^{14}C]D-glucose was expressed as pmol of glucose/mg of cellular protein. Each experiment was replicated three times (n=3) with 4 wells per condition per replication. All data were analyzed using all pups from each pregnant rat as an experimental unit. Data were ana-

lyzed for treatment differences by analysis of variance and intertreatment differences were determined by post-hoc test (Tukey's).

Figure 2. cAMP mediates Aß inhibition of neuronal glucose uptake. Abbreviations used for figure: C=control. Aß=amyloid ß-peptide. *a,* Neurons at day 4 post-plating were analyzed for cAMP content following exposure to Aß25-35 or scrambled control peptide for 2 hr, 2 days, or 3 days. A significant change in intracellular cAMP levels was observed in neurons exposed to Aß and this change was time-dependent. The highest cAMP level was found in neurons exposed to Aß for 2 hr. The cAMP level had fallen to the control level by 72 hr. *b-c.* Neurons at day 4 post-plating were assessed for glucose uptake and cellular protein at 0 and 1 hr following exposure for one hr to either Bt_2cAMP (0, 0.5, 1, 5, 10, 50 μM) or forskolin (0, 10, 25, 50, 100 μM). Neuronal glucose uptake was significantly inhibited by Bt_2 cAMP (*b*) or forskolin (*c*). This inhibitory effect was dose-dependent for both Bt_2 cAMP and forskolin. *d.* Since ßA25-35 elevated cAMP by 2 hr (*a*), neurons at day 4 post-plating were assessed for glucose uptake at 0 and 1 hr following exposure to either Aß25-35 or forskolin (10 μM) for 3 hr. Glucose uptake was significantly inhibited by Aß25-35, suggesting an acute inhibitory effect of Aß25-35.

METHODS. The cAMP content of cultured neurons was determined with the acetylation protocol of [125]I cAMP radioimmunoassay (Amersham). Each experiment was replicated three times (n=3) with 4 wells per condition per replication. All data were analyzed using all pups from each pregnant rat as an experimental unit. Data were analyzed for treatment differences by analysis of variance and intertreatment differences were determined by subsequent post-hoc test (Tukey's).

Figure 3. PKA mediates Aß inhibition of neuronal glucose uptake. Abbreviations used for figure: C=control, Aß=amyloid ß-peptide, PKAi=PKA inhibitor. Neurons at day 4 post-plating were analyzed for glucose uptake and cellular protein following exposure to KT-5720 (0.1 μM or 1 μM) in the culture medium and Aß25-35 or scrambled control peptide on cover slips for 24 hr. Glucose uptake by control neurons was 40 pmol/mg cellular protein. In contrast,

Aß25-35 significantly reduced glucose uptake to 15 pmol/mg cellular protein, i.e., 38% of the control neurons. This inhibitory effect of Aß25-35 was prevented by 1 µM KT-5720, but not by 0.1 µM KT-5720. This dose-dependent effect of PKA was highly significant. Each experiment was replicated three times (n=3) with 4 wells per condition per replication. All data were analyzed using all pups from each pregnant rat as an experimental unit. Data were analyzed for treatment differences by analysis of variance and intertreatment differences were determined by post-hoc test (Tukey's).
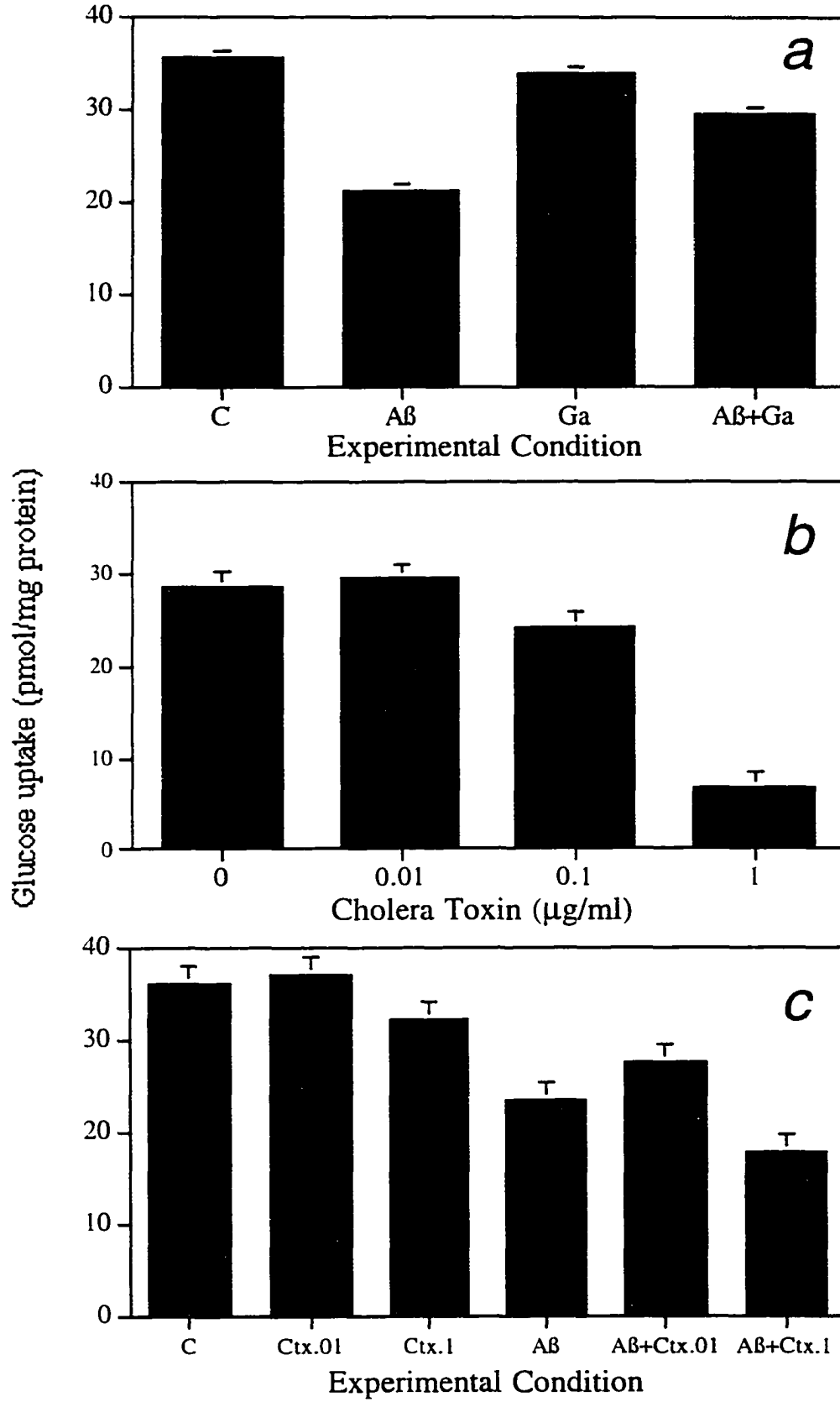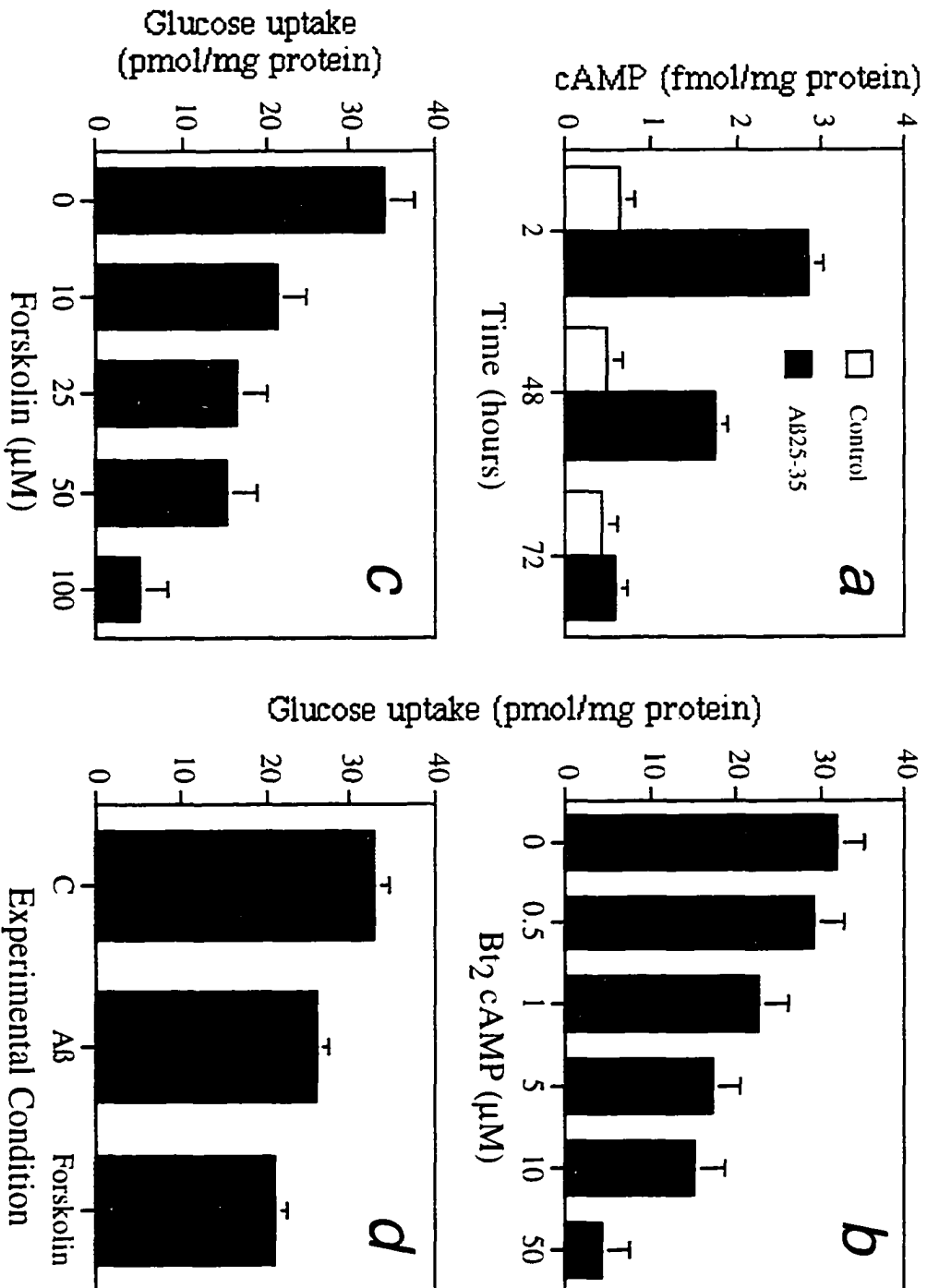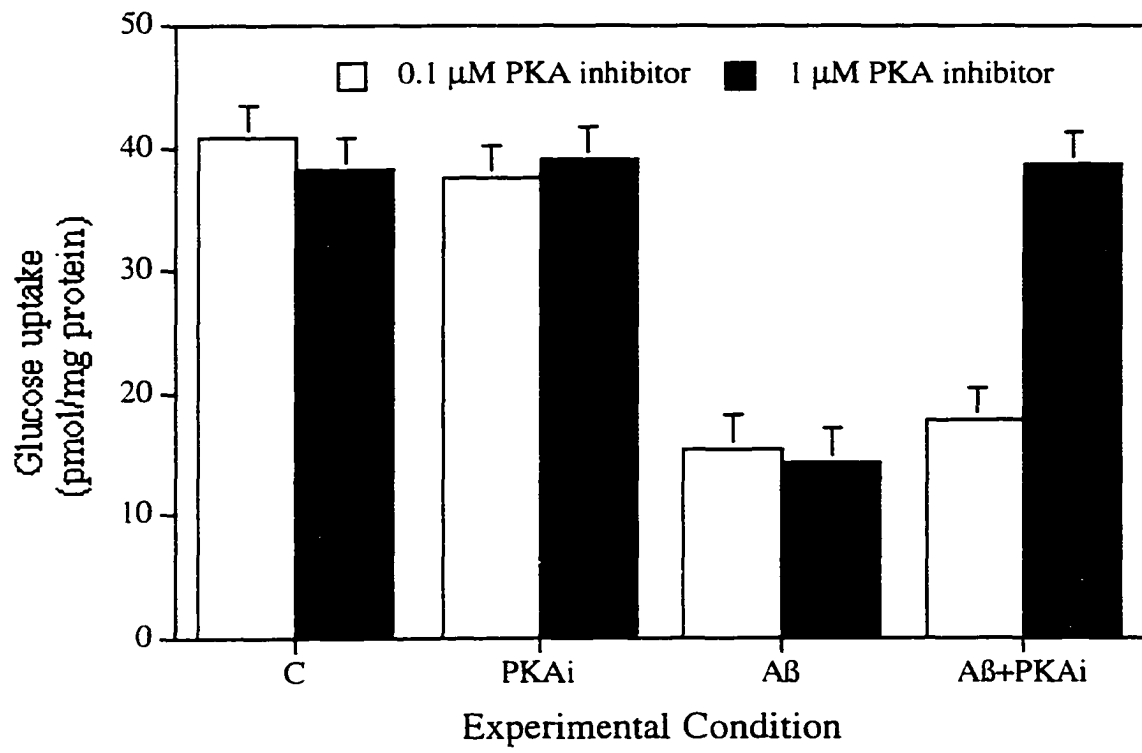
Figure 1

Figure 2

Figure 3

CHAPTER 3: EFFECTS OF INSULIN AND INSULIN-LIKE GROWTH FACTOR 1 (IGF-I) ON Aß INHIBITION OF NEURONAL GLUCOSE UPTAKE

A paper to be submitted to *Brain Research*

Teerasak Prapong, Etsuro Uemura, and Walter H. Hsu.

Abstract

Alzheimer's disease (AD) is characterized by deposition of amyloid ß-peptide (Aß). Aß has been shown to impair glucose uptake in cultured hippocampal neurons in vitro. However, how Aß inhibits glucose uptake is not known. We examined the effects of insulin and insulin-like growth factor 1 (IGF-I) on the inhibitory effect of Aß on neuronal glucose uptake. Insulin and IGF-I elevated neuronal glucose uptake, but the effect of IGF-I was more potent than insulin. Neurons exposed to Aß25-35 showed 38% less glucose uptake than the control. However, IGF-I and insulin prevented this inhibitory effect of Aß. To study the signaling pathway of IGF-I that stimulates glucose uptake, hippocampal neurons were studied for their glucose uptake following exposure to a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002) or a mitogen-stimulated protein kinase (MAPK) inhibitor (PD-98059). The stimulatory effect of IGF-I on neuronal glucose uptake was significantly inhibited by LY294002, but not by PD98059. The results suggest that (1) both IGF-I and insulin are effective in elevating neuronal glucose uptake and preventing the inhibitory effect of Aß and (2) PI3K, but not MAPK, plays a regulatory role in neuronal glucose uptake.

Introduction

The neuropathology of AD is characterized by deposition of Aß, a 39-43 amino acid protein [61]. Aß is the primary protein component of neuritic plaques [38], the morphology of which varies from diffuse deposits of nonfibrillar Aß to fibrillar Aß. Although there are numerous reports linking Aß to neurotoxicity *in vitro* and *in vivo* [39], the mechanisms involved in

Aß-mediated neuronal degeneration are not well understood. In vitro study has shown that Aß impairs glucose uptake in cultured hippocampal neurons [37] and astrocytes [43]; however, it is not known whether Aß affects neuronal and astrocytic glucose uptake in AD patients. There is some evidence suggesting that glucose metabolism is changed in the brains of AD patients [14,29,46,48]. It was calculated that reduction of cerebral glucose utilization ranges from 19% in mild cases to 40% and more in severely diseased patients [27,34]. Alzheimer's patients have abnormally low blood glucose [28] and glucose has been shown to enhance memory in Alzheimer's patients and normal elderly adults [8,32]. Because glucose infusion is usually associated with elevation of plasma insulin, a group of Alzheimer's patients was further assessed by raising plasma insulin, but preventing a rise in plasma glucose [9]. There was a striking memory enhancement in these patients, suggesting a functional importance of insulin in the brain. Reduced cerebral glucose uptake by individuals genetically at risk for developing AD also supports the notion that glucose deprivation precedes neuronal degeneration [31,45,47].

Neurons exposed to Aß become more susceptible to glucose deprivation [7]; however, IGF-I and IGF analogues are effective in promoting cell survival under this condition [6,22]. Furthermore, IGF-I is shown to protect, and more importantly, to rescue rat hippocampal neurons against Aß-induced toxicity [13]. The protective mechanism of IGF-I is not known, but it was shown that insulin and IGF-I act as a neuronal growth factor [53,57]. In transgenic mice that overexpress IGF-I in the brain, glucose uptake increases significantly in the brain [20]. *In vitro* studies have also shown that IGF-I increases glucose uptake in skeletal muscle, mesangial cells, L6 myotubes, and PC12 cells [17,23,35,60]. Although several studies in humans suggest the importance of insulin in hippocampal energy metabolism [19,26] and memory facilitation [8,9], it is not known whether insulin and IGF-I promote glucose uptake by neurons. In the present study, the effects of insulin and IGF-I on glucose uptake were examined in hippocampal neurons exposed to Aß25-35 *in vitro*. The signaling pathways of insulin and IGF-I that stimulate neuronal glucose uptake were examined by exposing hippocampal neurons to a

phosphoinositide 3-kinase (PI3K) inhibitor or a mitogen-stimulated protein kinase (MAPK) inhibitor.

Material and Methods

Aß25-35, a neurotoxic portion of Aß peptide that corresponds to amino acids 23-35 of Aß found in AD [61] and scrambled peptide [40]were synthesized and purified at the University of Iowa Protein Facility. The scrambled peptide has the following sequence: H-Ile-Met-Leu-Gly-Asn-Gly-Ala-Ser-Ile-Gly-OH. Scrambled peptide and Aß25-35 were dissolved in Millipore-filtered double distilled water (pH 7.2, 10 mg/ml) and aliquots were stored at -20 °C. The working concentrations of each peptide (10-100 μg/ml) were prepared by further dilution in double-processed tissue culture water. Rat hippocampal neurons were prepared by modifying the method previously described by [1]; however, we used N2 instead of N1 supplement in our culture. E17 - E18 pregnant rats (Holtzman) were euthanized in the halothane chamber. The fetuses were removed by cesarean section. Then, the brains were dissected and put into cold Earles Balance Salt Solution (EBSS) by sterile technique. Hippocampi were dissected under the stereoscope and placed into cold sterile EBSS. The tissues were incubated in a 0.2% trypsin EBSS solution for 15 minutes and in a 0.2% trypsin inhibitor EBSS solution for 5 minutes, respectively. The tissue was dissociated by tituration through a fire-polished pasteur pipette in Minimum Essential Medium (MEM) supplemented with 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 2 mM $NaHCO_3$, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1 ml of N-2 supplement (Gibco). Tissue suspension with a density of 1-3 x 10^5 cells/ml in MEMN2 media was plated in 24 -well tissue culture plates coated with polyethylenelmine (PEI) alone or re-coated with either Aß25-35 or scrambled peptide. The culture was incubated at 37 °C in 5% CO_2 humidified atmosphere for 4 days before treatment.

To study the effect of Aß, IGF-I, and insulin on neuronal glucose uptake, cells were exposed, at day 4 post-plating, to 10 μM Aß25-35 coated on cover slips, 50 nM IGF-I, or 50 nM

insulin for 24 hr. Cover slips coated with Aß25-35 were prepared by allowing 50 μl of Aß25-35 placed on a round cover slip (12 mm in diameter) to air dry. The coated-side of the cover slip was placed on top of cultured neurons. Neuronal uptake of glucose was assayed by using D-[U-^{14}C]glucose (313 mCi/mmol, ICN) at final glucose concentration of 600 nM (0.1 μCi/500 μl media in well). Cell lysates were used for determination of radioactivity by using a beta liquid scintillation counter (Packard, 1600TR) and for assaying of total cell protein (Bio-Rad protein assay kit). Neuronal uptake of [^{14}C] D-glucose was expressed as pmols of glucose/mg of cellular protein. To examine the signaling pathway of IGF-I that elevates neuronal glucose uptake, hippocampal neurons at day 4 post-plating were exposed to 50 nM IGF-I or 50 nM insulin for 24 hr, in the presence of either 50 μM PI3K inhibitor (LY294002, Eli Lilly) or 30μM MAPK inhibitor (PD-98059, Biomol). Each experiment was replicated three times (n=3) with 4 wells per condition per replication. All data were analyzed using all pups from each pregnant rat as an experimental unit. Data were analyzed for treatment differences by analysis of variance and intertreatment differences were determined by subsequent post-hoc test (Tukey's).

Results

The effects of insulin and IGF-I on glucose uptake were examined in hippocampal neurons exposed to Aß25-35. Hippocampal neurons responded significantly to Aß25-35, IGF-I, and insulin [$F(5,12)=72.9$, $p=0.0001$] (Fig. 1). Neuronal glucose uptake was increased by either IGF-I ($t=-6.78$, $p=0.0001$) or insulin ($t=-3.29$, $p=0.006$), but the effect of IGF-I was more potent than insulin ($t=3.49$, $p=0.0045$). Aß25-35 inhibited the glucose uptake by 38% compared with the control ($t=10.84$, $p=0.0001$). Both IGF-I and insulin prevented this inhibitory effect of Aß25-35, but the preventive effect of IGF-I was more potent than that of insulin ($t=3.47$, $p=0.0046$). Thus, neurons exposed to both Aß and IGF-I showed significantly more glucose uptake than the control ($t=-2.81$, $p=0.016$), while those cells exposed to both Aß and insulin had glucose uptake similar to the control. The results suggest that both IGF-I and insulin are effective in

elevating neuronal glucose uptake and preventing an inhibitory effect of Aß.

Since both IGF-I and insulin significantly elevated neuronal glucose uptake, the effects of a PI3K inhibitor (LY294002) and a MAPK inhibitor (PD-98059) were assessed on the stimulatory effect of IGF-I on glucose uptake. The effect of 50 µM LY294002 on glucose uptake is shown in Fig. 2. PI3K inhibitor alone had no effect on neuronal glucose uptake; however, it significantly prevented the stimulatory effect of IGF-I on glucose uptake ($t=2.85$, $p=0.02$). The effect of PD-98059 is shown in Fig. 3. The MAPK inhibitor alone did not affect neuronal glucose uptake; however, unlike the PI3K inhibitor, the MAPK inhibitor did not prevent the stimulatory effect of IGF-I on glucose uptake. The results suggest that PI3K, not MAPK, mediates the elevation of glucose uptake by IGF-I.

Discussion

The present study has shown that both IGF-I and insulin are effective in elevating glucose uptake by cultured hippocampal neurons. In both skeletal muscle and adipocytes, insulin and IGF-I were shown to control the actual number of GLUT4 in the plasma membrane by stimulating translocation of GLUT4 [36,44,59]. Similarly, insulin and IGF-I were shown to translocate GLUT3 in L6 myotubes [41]; however, what regulates translocation of neuronal GLUT3 is not known at present. Insulin and IGF-I share structural and functional features, including an α-subunit that binds the hormone-like agent and a ß-subunit that has a tyrosine-specific protein kinase [56]. The metabolic effects of these hormones are initiated by their binding to the extracellular domain of the specific membrane receptors, which lead to the activation of, among others, Ras and PI3K [21,54]. Many of these kinases appear to be arranged in cascades, including a cascade that results in the elevation of glucose uptake in insulin sensitive cells [55]. The signaling pathways that stimulate neuronal glucose uptake are not known. In adipocytes, PI3K was shown to increase GLUT4 translocation to the plasma membrane and glucose transport [16,24,30,33]. Furthermore, a PI3K inhibitor, LY294002, was shown to in-

hibit glucose uptake [50] by interfering with insulin mediated translocation of GLUT4 [5]. Similarly, our study demonstrated that LY294002, but not MAPK inhibitor, inhibits IGF-I stimulated neuronal glucose uptake. This suggests that IGF-I stimulated increase in glucose transport by hippocampal neurons involves a signaling pathway of PI3K.

Those neurons exposed to Aß25-35 for 24 hr showed 38% less glucose uptake compared with the control. Thus, our data confirms the previous study [37)]. The present study has also shown that insulin and IGF-I prevent this inhibitory effect of Aß, elevating the glucose uptake either to the control levels by insulin or more than control levels by IGF-I. In healthy, nonstarved mammalian brain, glucose is the only substrate for the formation of energy in the form of ATP [15]. Neurons with impaired glucose uptake or exposed to Aß have impaired mitochondrial activity and suppressed production of ATP [39]. How these *in vitro* data relate to the brains of AD patients is not certain, but it was estimated, based on the rates of oxidized glucose and oxygen consumption, that the rate of cerebral ATP formation decreases by 7% in early-onset AD and 20% in late-onset AD [25]. Thus, the capacity of insulin and IGF-I to promote glucose uptake and prevent Aß inhibition of neuronal glucose uptake is of importance to normal and pathological brain functioning. An increasing body of evidence suggests that insulin has a vital role in brain function [58]. Expression of the insulin gene and insulin synthesis occur in a highly specific pattern, with the highest density in hippocampal pyramidal neurons [12]. Plasma insulin was also shown to cross the blood-brain barrier by saturable receptor-mediated transcytosis, and residual insulin is removed through the CSF [2,3,52]. At present, normal insulin and IGF-I levels in the human brain are not known. However, a deficit in these hormones is suspected in the brains of AD patients because of their higher plasma insulin and IGF-I, and lower CSF insulin levels [10]. Insulin and IGF-I receptors are also present in the human brain [42,49,51], and their numbers increase in the brains of AD patients [4,11,18]. If this proliferation of insulin and IGF-I receptors is the brain's attempt to compensate for a lack

of insulin and IGF-I, the reduced cerebral glucose utilization found in AD may reflect the cerebral insulin and IGF-I deficit in AD [27].

Acknowledgements

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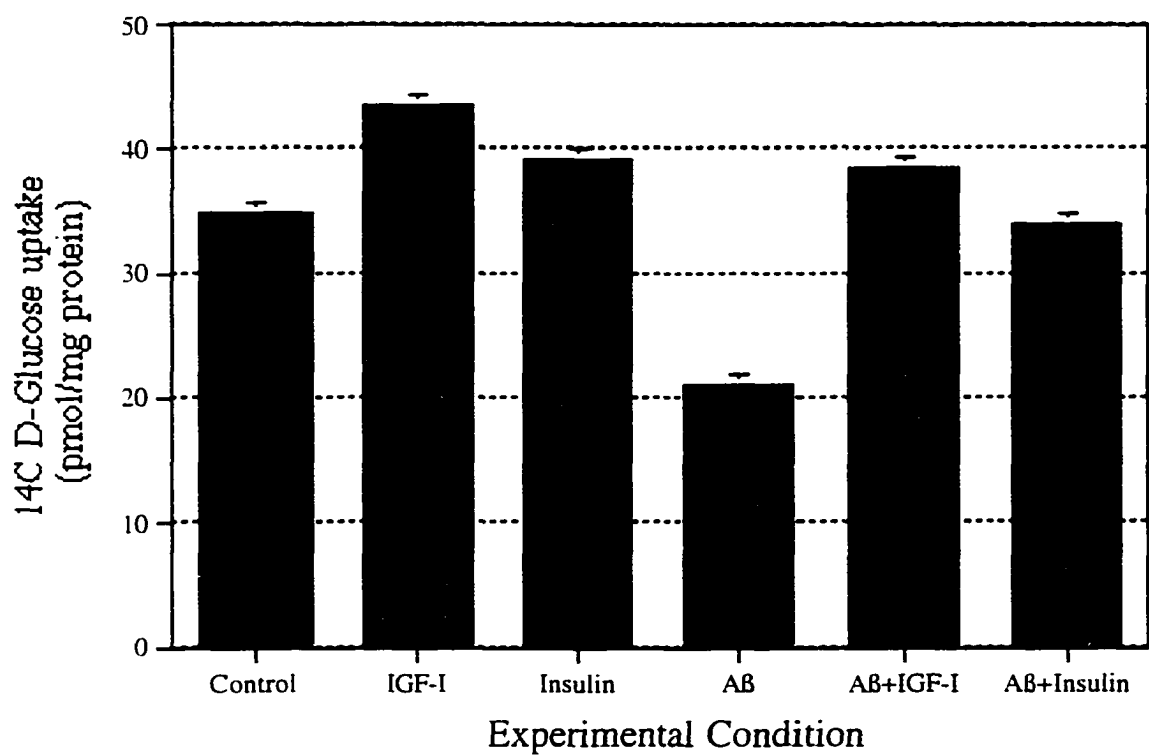
Figure 1. The effects of insulin and IGF-I on glucose uptake in hippocampal neurons exposed to Aß25-35.
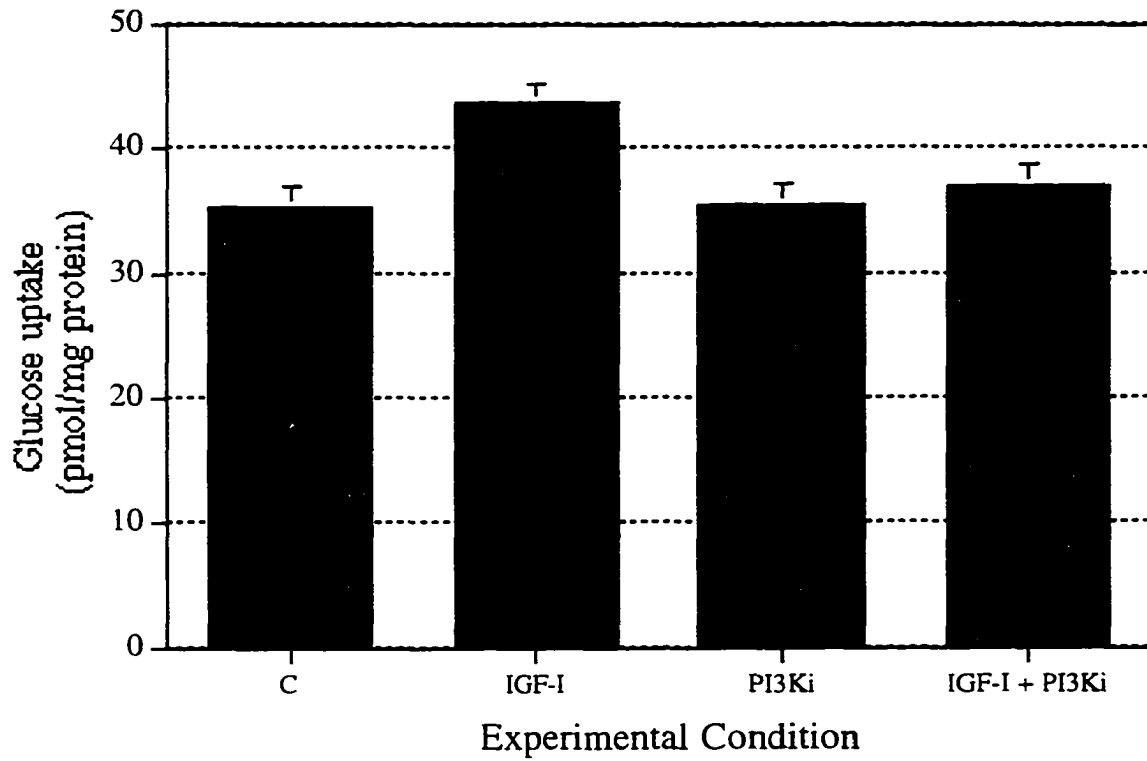
Figure 2. The effect of a PI3K inhibitor (LY294002) on the stimulatory effect of IGF-I on neuronal glucose uptake.
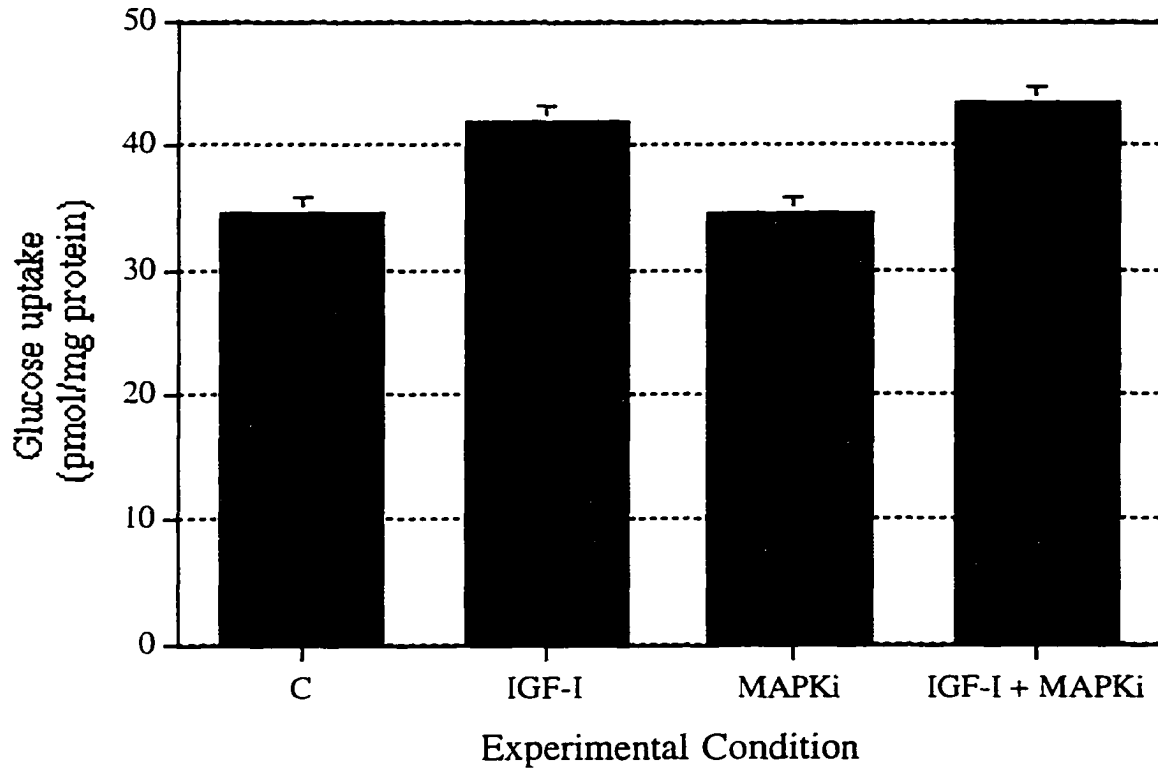
Figure 3. The effect of a MAPK inhibitor (PD-98059) on stimulatory effect of IGF-I on neuronal glucose uptake.

CHAPTER 4: GENERAL DISCUSSION

The research in this dissertation was to test the hypothesis that (1) G protein and cAMP mediate Aß inhibition of neuronal glucose uptake. (2) Insulin and IGF-I prevent the inhibitory effect of Aß on neuronal glucose uptake. and (3) either IP3K or MAPK mediates the effect of IGF-I (Figure 1). The results from the studies suggested that (1) the inhibitory effect of Aß (Aß25-35) on glucose uptake by culture hippocampal neurons is mediated via a G protein and cAMP-dependent PKA pathway, (2) both insulin and IGF-I induce elevation of glucose uptake and prevent the inhibitory effect of Aß, and (3) IGF-I stimulates glucose uptake via IP3-Kinase.

To test the first hypothesis, G Protein antagonist-2 (GPAnt–2) was used to block the binding of the receptor with G protein [44]. The GPAnt–2 minimized the inhibitory effect of Aß25-35 on neuronal glucose uptake, suggesting that G protein plays a key role in Aß decreasing glucose transport of neurons. It is known that cholera toxin activates G protein activity resulting in more cAMP in the cells [30]. Cholera toxin in the present study not only inhibited neuronal glucose uptake, but also potentiated the inhibitory effect of Aß. Based on these data, cAMP and PKA were speculated as two molecules involved in the pathway. By using dibutyryl cAMP (Bt$_2$cAMP) and forskolin, it was concluded that they have an inhibitory role in neuronal glucose uptake further supporting the involvement of cAMP. In the cAMP-dependent protein kinase pathway, cAMP exerts its action on cAMP-dependent protein kinase or PKA [45]. The present study shows that PKA inhibitor can prevent the inhibitory effect of Aß on neuronal glucose uptake. This could be the reason for the attenuation effect of PKA on Aß toxicity on cultured rat cerebral neurons shown by Ueda et al. [59].

The present study demonstrates that G protein and cAMP mediate Aß25-35 inhibition of neuronal glucose uptake. How these *in vitro* data relate to the decreased glucose metabolism in AD is not certain. It is possible that the decreased cerebral glucose uptake shown in AD may reflect, in part, the inhibitory effect of Aß on neuronal and astrocytic glucose uptake. Such an
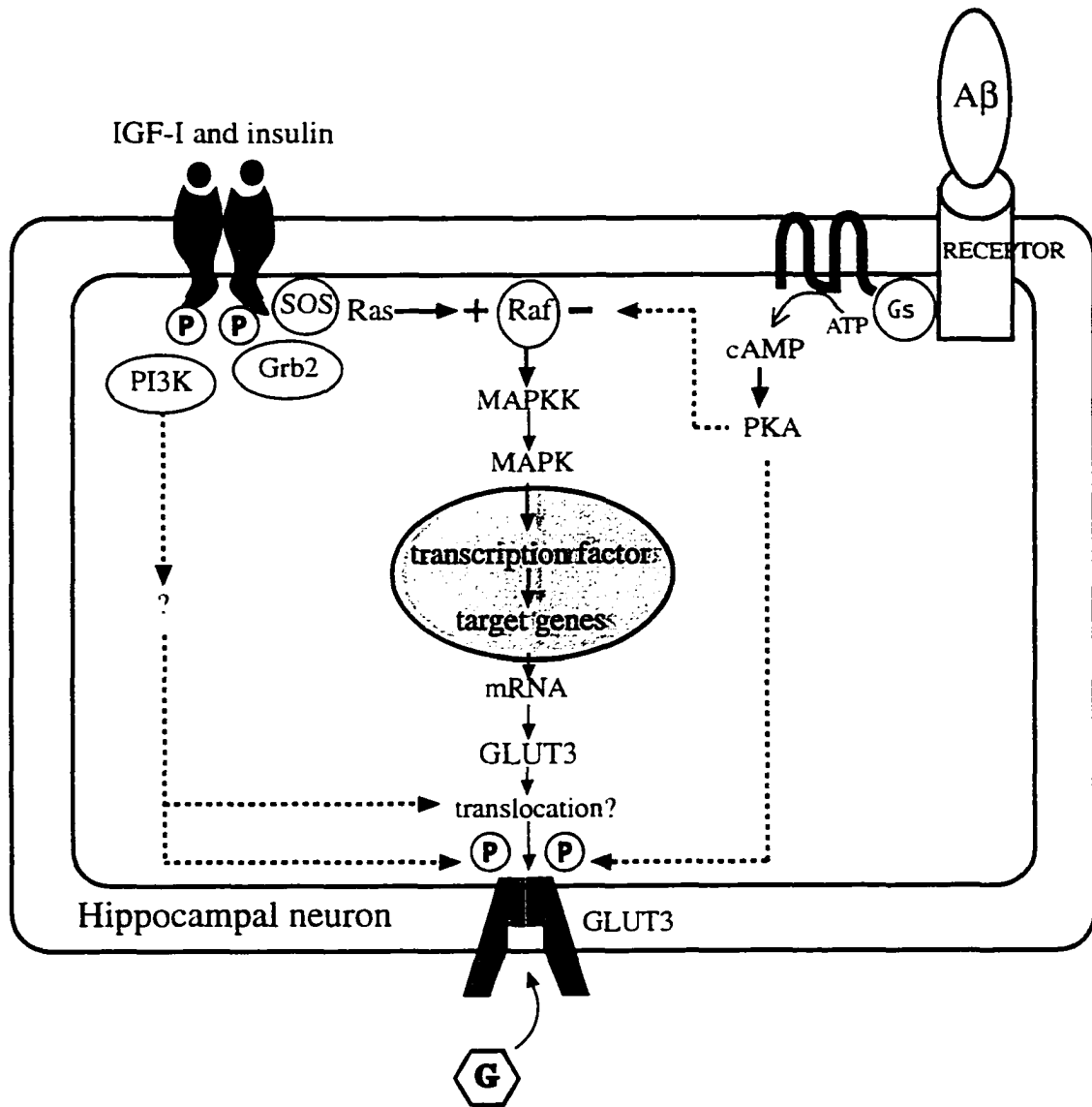
Figure 1. Hypothetical pathways mediating the inhibitory effect of Aβ and the stimulatory effect of insulin and IGF-I on neuronal glucose uptake

hypothesis infers the presence of functional G protein-stimulated activity of adenylyl cyclase and cAMP in AD patients. Studies from brain tissues of AD patients revealed that $G\alpha$-subunit is unchanged [42] or even elevated Gs α-mRNA [26]. Catalytic activity of cAMP and cAMP-dependent protein kinase was also reported as normal [9,22,43,49]. Nevertheless, other studies indicated the opposite [16,53]. This inconsistency may reflect differences in postmortem instability of the enzyme at different stages of Alzheimer's disease [9].

The present study assessed intracellular cAMP following exposure to Aß25-35 for 2 hrs to 72 hrs. Interestingly, chronic exposure to Aß resulted in a decrease of cAMP that may be due to desensitization of G protein-coupled receptors. The mechanism of desensitization of G protein-coupled receptors was reviewed: it was found that tachykinin, M2 muscarinic and nicotinic receptors can be desensitized [15]. The tachykinin receptor was reported to be a site for Aß binding [14,66]. More over, nicotinic receptors and M2 muscarinic receptors are decreased in AD patients [41,54]. Both cAMP and PKA also play a role in the process of desensitization [15]. It was proposed here that in the early state of decreasing glucose uptake caused by Aß, cAMP and PKA might be key molecules affecting the intrinsic activity of GLUT3. Consequently, rising cAMP and PKA activity leads to desensitization of receptors for Aß binding which would lead to a decline of cAMP. The present study showed that the cAMP level is inversely related to the rate of glucose uptake. Decreased cAMP levels as a result of desensitization of G protein-coupled receptors should make the rate of glucose uptake return to normal. However, it did not. It is possible that Aß-mediated elevation of cAMP suppressed GLUT3 mRNA, and the subsequent decrease in number of GLUT3 available for glucose transport. In 3T3-L1 adipocytes, increasing cAMP by either forskolin or 8-bromo-cAMP was shown to repress expression of the GLUT4 gene [32]. However, GLUT3 mRNA levels in neurons chronically exposed to Aß is not known.

The second study was to test the hypothesis that insulin and IGF-I prevent the inhibitory effect of Aß on glucose uptake and either PI3K or MAPK mediates the effect of IGF-I. The

brains of humans and rodents were shown to have receptors, mRNAs and binding proteins for insulin and IGFs [1-5,7,8,10,13,19,24,27,29,33,37-40,46,48,51,52,56-58,62,64,65]. Recent studies found alteration of insulin and its receptors in AD patients [23]. The patients have high levels of plasma insulin and IGF, whereas their CSF levels are lower than non AD people of the same age [18]. In addition, insulin and glucose were shown to improve memory in AD patients [17,35], and injecting streptozotocin into the rat brain was shown to impair memory due to desensitization of insulin receptors [31,47]. IGF-I, which has a structure and receptors similar to insulin was shown to prevent Aβ toxicity and increase glucose uptake in PC12 cells in vitro [20,65]. Whether and how insulin and IGF exert their function on neuronal glucose uptake *in vivo* is not known.

The present study showed that both insulin and IGF-I elevate glucose uptake in the cultured neurons. The result agrees with the previous study in PC12 cells [65]. IGF-I and IGF-II were reported to have their protective action against calcium-mediated hypoglycemic damage in cultured hippocampal and septal neurons [12]. However, the study did not look for the effect of IGFs on normal glucose uptake. The hippocampus is one of the prominent areas in the brain having IGF-I receptors [29]. This suggests why IGF-I has more potency to increase glucose uptake in the cultures used in this study. Nevertheless, how insulin and IGFs regulate neuronal glucose uptake is not known. It was demonstrated in L6 myotubes that insulin and IGF-I increase translocation of GLUT3 from the cytosol pool to plasma membrane[6,63]. However, the translocation of GLUT3 in neurons has not been demonstrated at present. The present study showed that both IGF-I and insulin were able to prevent the inhibitory effect of Aβ25-25 on glucose uptake, although IGF-I is more potent than insulin. Insulin was shown to increase GLUT3 mRNA in rat brain under chronic insulin hypoglycemia [60], suggesting the involvement of insulin in cerebral glucose metabolism.

The present study also investigated the pathway used by IGF-I to regulate neuronal glucose uptake. The results showed that a IP3K inhibitor (LY294002), but not MAPK inhibitor

(PD98059), prevents the stimulatory effect of IGF-I on neuronal glucose uptake. This suggests that IGF-I modulates the glucose uptake via a PI3K pathway. The metabolic effects of IGF-I and insulin are initiated by their binding to the extracellular domain of specific membrane receptors, which lead to the activation of, among others, Ras and PI3K [25,55]. Many of these kinases appear to be arranged in cascades, including a cascade that results in the elevation of glucose uptake in insulin sensitive cells [61]. The signaling pathways that stimulate neuronal glucose uptake are not known. In adipocytes, PI3K was shown to increase GLUT4 translocation to the plasma membrane and glucose transport [21,28,34,36]. Furthermore, a PI3K inhibitor, LY294002, was shown to inhibit glucose uptake [50] by interfering with insulin mediated translocation of GLUT4 [11]. Similarly, the present study demonstrated that LY294002, but not a MAPK inhibitor (PD98059), inhibits IGF-I stimulated neuronal glucose uptake. This suggests that IGF-I stimulated increase in glucose transport by hippocampal neurons involves a signaling pathway of PI3K.

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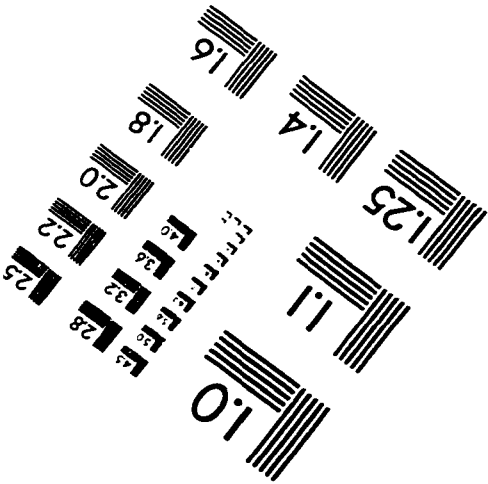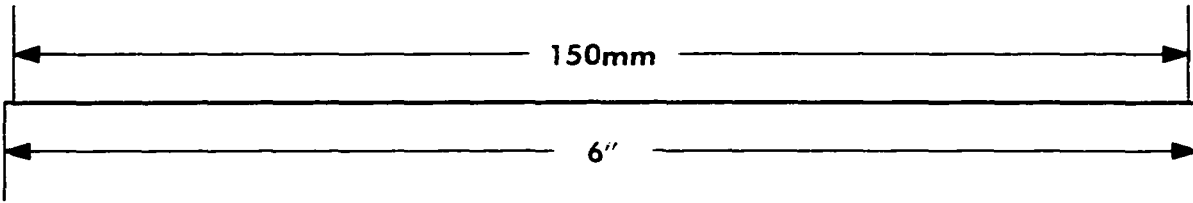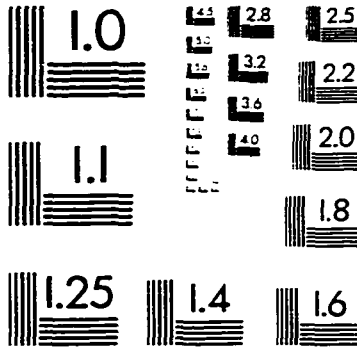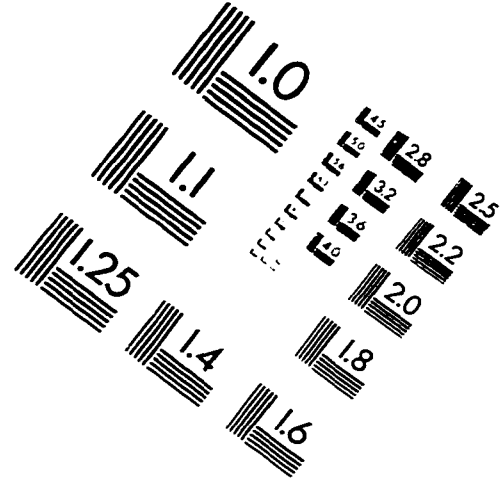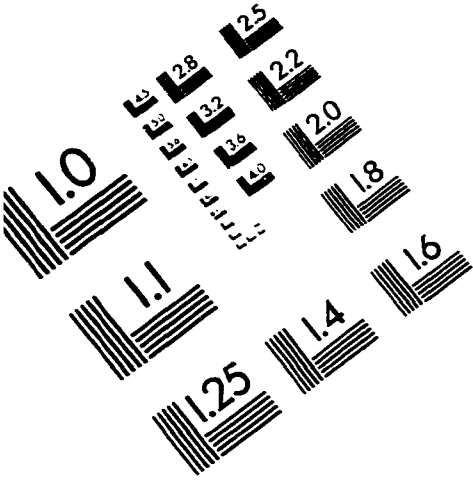
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ACKNOWLEDGEMENTS

IMAGE EVALUATION
TEST TARGET (QA-3)