NEURAL SUBSTRATES OF INSULIN-MEDIATED MEMORY FACILITATION IN EARLY ALZHEIMER'S DISEASE; THE IMPACT OF THE APOLIPOPROTEIN E-EPSILON-4 ALLELE ON HIPPOCAMPAL INSULIN RESPONSES

BY

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Abstract

Background: Several studies have demonstrated that insulin delivered by nasal spray acutely improves cognitive performance in early Alzheimer's disease. Furthermore, the apolipoprotein E-epi\silon-4 allele, a known AD risk factor, appears to influence those cognitive responses in a dose dependent manner, with carriers exhibiting impaired cognitive performance at higher insulin doses. The neural correlates of this phenomenon are however presently undefined. Hippocampal neurons are known to express insulin receptors in high densities, and neuronal insulin signaling is thought to modulate long-term potentiation mechanisms, suggesting that insulin might act at the hippocampus to generate cognitive enhancement. How the apolipoprotein E-epsilon-4 allele might interact with hippocampal insulin responses is not clear however.

Methods: We studied the effects of a single 40IU dose of insulin aspart on hippocampal activation in 10 cognitively intact and 18 early AD subjects in a double-blind, counterbalanced, crossover memory encoding BOLD-based functional MRI study. We also assessed the effects of insulin on non-cognitive brain function with an fMRI motor task, and mean cerebral blood flow with arterial spin-labeled MRI. Last, insulin's effect on cognitive performance was assessed. We

hypothesized AD subjects would demonstrate insulin-related increases in hippocampal activation, and that insulin-related changes would be greatest in apolipoprotein E-epsilon-4 negative AD. We further hypothesized insulin-related increases in mean cerebral blood flow in all subjects.

Results: We observed an apolipoprotein E-epsilon-4 based interaction in insulin-related changes in hippocampal activation in AD, with carriers exhibiting decreased activation with insulin. Mean cerebral blood flow did not change significantly with insulin administration. Cognitive performance increased in the apolipoprotein E-epsilon-4 negative AD subjects after insulin administration.

Conclusions: There is growing body of research indicating that faulty insulin signaling might be a component of AD neuropathology. The results herein are the first description of the neural correlates of insulin-related cognitive enhancement in AD, and provide direct evidence that insulin might serve an important neuromodulatory role in AD. Insulin also appears to modulate non-cognitive functions in a manner similar to hippocampal activity, suggesting that insulin might have wide spread effects on brain physiology, while the arterial spin-labeled MRI results suggest that insulin's effects are independent of cerebral blood flow. Our

results furthermore underscore the importance of the apolipoprotein E-epsilon-4 allele in brain insulin responses.

List of Tables and Figures

Chapter 1-Introduction

There is no cure at the moment for Alzheimer's disease, and the few treatments available do not demonstrate long-term effectiveness. The prevalence of Alzheimer's disease increases with age, doubling every 5 years over age 65. Increased life expectancy in the US and other developed countries has contributed to an unprecedented growth in the elderly population and a dramatic increase in AD incidence. There are an estimated 4.5 million people in the US and 20 million worldwide with AD (World Health Organization). The cost of AD treatment in the US is estimated to be $100 billion annually and is rapidly rising. Medicare spending on Alzheimer's disease is expected to grow to $49.3 billion, a 54% increase over 2000 costs. Medicaid spending is expected to grow to $33 billion, an 80% increase over costs in 2000. (Kawas & Katzman, 1999) It has been estimated that delaying the onset of AD by just 5 years could reduce annual health care costs in the US by $50 billion, (Kawas & Katzman, 1999) underscoring the importance of developing effective strategies for the treatment and prevention of AD.

AD Neuropathology

While the definitive cause of Alzheimer's disease is presently unclear, most research currently focuses on altered amyloid processing and tau phosphorylation as primary etiologic factors. Beta-amyloid appears to be a naturally occurring product

of amyloid precursor protein (APP) in both neurons and astrocytes. Amyloid precursor protein degradation may follow either of two pathways. The more common, non-amyloidogenic pathway is characterized by the initial cleavage of APP by alpha-secretase, yielding a soluble N-terminal fragment, and a membrane-bound C-terminal fragment. Gamma-secretase next processes the C-terminus fragment into a small soluble fragment (P3) as well as an additional membrane-bound fragment. (Minati, Edginton, Bruzzone, & Giaccone, 2009)

The amyloidogenic degradation of APP begins with cleavage by beta-secretase at a site different from that cleaved by alpha-secretase, resulting in a smaller soluble N-terminus fragment and a larger membrane-bound C-terminus fragment. Gamma-secretase, the activity of which is modulated by glycogen synthase kinase-3 alpha, (GSK-3a) (Phiel, Wilson, Lee, & Klein, 2003) then cleaves the C-terminus fragment into a membrane bound polypeptide and an insoluble N-terminus fragment of either 40 or 42 amino acid residues in length. The plaques seen in AD are composed primarily of the 42 amino acid product, known as Abeta-42.

The neurofibrillary tangles associated with AD are composed of hyperphosphorylated structural protein tau. Tau phosphorylation is a normal cellular process that stabilizes cellular structure by promoting the binding of tau to

microtubules. Hyperphosphorylated tau however self aggregates, forming neurofibrillary tangles characteristic of AD. Tau phosphorylation is regulated by at least 3 proline-dependent kinases, including GSK-3β, as well as specific phosphatases. (Muyllaert, et al., 2008; Muyllaert, et al., 2006) Dysregulation of tau kinase/phosphatase activity is thought to contribute to neurofibrillary tangle formation in AD. The factors that modulate the balance between these kinases and phosphatases in AD are not clear.

Insulin and AD Neuropathology

A growing body of research suggests that alterations in insulin signaling might contribute to AD pathology. Key steps in the production of both Abeta42 and tau involve GSK-3 activity. Inhibition of GSK-3a activity modulates gamma secretase function, reducing Abeta production. (Phiel, et al., 2003) Similarly, inhibition of GSK-3b leads to reduced tau phosphorylation. (Planel, et al., 2007) Insulin signaling promotes the phosphorylation and inactivation of GSK-3. (Boron & Boulpaep, 2003) Insulin signaling therefore appears to be intimately linked to the production of both Abeta and neurofibrillary tangles, and might therefore modulate key steps in AD pathogenesis. Insulin furthermore competes with Abeta for degradation by insulin degrading enzyme (IDE) (I. V. Kurochkin, 2001; Igor V.

Kurochkin, 2001; McDermott & Gibson, 1997; Qiu, et al., 1998) and higher insulin levels might therefore inhibit Abeta clearance.

Post-mortem examination of AD brain tissue shows decreased insulin receptor density and tyrosine kinase activity in AD relative to age-matched controls, (Frolich, Blum-Degen, Riederer, & Hoyer, 1999) suggesting impaired insulin action as a component of AD pathology. Furthermore, decreased insulin, insulin-like growth factor, as well as decreased mRNA for insulin receptor substrate (IRS) and several insulin signaling intermediates, along with increased GSK-3b activity have been described in AD brain. (Steen, et al., 2005) In total, these reports indicate altered insulin signaling mechanisms in AD, and have led to the term "type 3 diabetes" (Steen, et al., 2005) to describe the changes in brain insulin signaling in AD.

Streptozotocin (STZ) is used experimentally to inhibit insulin signaling mechanisms, and STZ-treated mice exhibit increased tau phosphorylation that is partially reversible with insulin administration. (Schechter, Beju, & Miller, 2005) Cerebrospinal fluid Abeta42 concentration exhibits a negative correlation with the number of neuritic plaques in cortex and hippocampus is AD, (Strozyk, Blennow, White, & Launer, 2003) and lower CSF level of Abeta42 in relation to Abeta40 has

been shown to be highly predicative of AD development. (Graff-Radford, et al., 2007) Experimental insulin administration has been shown to increase CSF Abeta42 in AD, (G. Watson, et al., 2003) and lead to increased plasma Abeta40 concentration, (M. A. Reger, et al., 2007) suggesting that insulin might modulate amyloid metabolism. It appears therefore that insulin signaling mechanisms might be intimately linked to the expression of the classic neuropathologic hallmarks of AD, and that modulation of insulin signaling might modify that expression of AD neuropathology and possibly pathologic burden.

Insulin and Brain Structure

Magnetic resonance imaging is commonly used to assess brain volume as a reflection of pathologic burden. We earlier described a positive correlation between whole brain volume obtained from MRI data and peripheral insulin levels during a 3-hour I.V. glucose tolerance test. (Burns, et al., 2007) These findings suggest that chronic insulin levels influence brain structure, and might reflect insulin's proposed influence on AD pathologic burden. With the knowledge that insulin receptors are found in high density in the hippocampus, we narrowed this analysis focusing on the relationship between peripheral insulin and hippocampal volume. The results of this preliminary study, presented in Chapter 2, demonstrate a positive correlation

between peripheral insulin levels and limbic gray matter volume including the hippocampus in AD. Thus, higher long-term insulin exposure to insulin appears to be associated with preserved hippocampal volume in AD. While speculative, insulin might act to inhibit the accumulation of, or promote the clearance of amyloid and/or tau in hippocampus in AD.

It is important to note that insulin degrading enzyme (IDE) metabolizes both insulin and Abeta. (Farris, et al., 2003; Qiu, et al., 1998) While insulin might possess neurotrophic or protective properties, chronically elevated insulin levels might paradoxically promote Abeta deposition by competitively inhibiting Abeta degradation by IDE. PPAR gamma agonists appear to up-regulate IDE transcription, (Du, et al., 2009) and have been shown to improve cognitive function in AD as well, (Pedersen, et al., 2006; Risner, et al., 2006; G. Watson, et al., 2005) suggesting that this class of drug might circumvent the potential negative effects of chronic insulin administration on Abeta degradation and offer novel therapeutic opportunities for AD patients.

Insulin and Memory

The majority of brain insulin is formed in the pancreas and traverses the blood-brain barrier to reach the central nervous system, (Banks,

2004) where insulin receptors are found in high density in specific regions including the olfactory bulbs, hypothalamus and the hippocampus. Several reports of memory enhancement with insulin in AD suggest that insulin may be involved in cognitive processes. Craft et al studies the effects of experimentally induced hyperinsulinemia in 23 early AD subjects and 14 cognitively intact older adults using a hyperinsuliinemic/euglycemic clamping technique. Story recall and selective attention were improved acutely when insulin levels were elevated, while holding glucose levels at baseline in AD. Memory performance did not however improve when glucose levels were raised while holding insulin at baseline levels. (S. Craft, et al., 1999) It appeares therfor that insulin levels can influence cognitve performance in AD independent of glucose.

Furthermore the effects of insulin on cognitive performance in early AD appear to differ according to apolipoprotein E-epsilon-4 genotype. Crafet et all showed that AD noncarriers specifically exhibit memory facuiitation with insulin while carriers do not using the euglycemic clamping method. (S. Craft, et al., 1999) This same study showed that glucose disposal also differed according to apolipoprotein E-epsilon-4 genotype, wu\ith AD noncarriers exhibiting less insulin-mediated glucose disposal than AD carriers or control subjects. Specifically how apolipoprotein E-epsilon-4 genotype influences cognitive enhancement in AD is not clear, but it might be that epsilon-4 genotype is related to oxidative metabolism.

More recently, insulin delivered via nasal spray has been shown to improve memory similar to IV administration, without the risk of hypoglycemia. Reger et al showed that a single dose of insulin delivered via nasal spray facilitated recall in 13 early apolipoprotein E-epsilon-4 negative AD subjects , while carriers did not respond at a 20IU dose and performed below baseline after a 40IU dose of insulin.(M. Reger, et al., 2006)

Plasma glucose and insulin levels do not appear to change significantly after intranasal administration of insulin. (M. Reger, Watson, Green, Baker, et al., 2008; M. Reger, Watson, Green, Wilkinson, et al., 2008; M. A. Reger & S. Craft, 2006; M. A. Reger, et al., 2006) These studies collectively demonstrate improved performance during memory encoding tasks, commonly word and story recall, when tested within about 15 minutes of insulin administration., suggesting that the nasal route of administration is a safe and effective method to study the acute effects of insulin on cognitive function. Rat studies have shown that IGF-1, structurally similar to insulin, might access the CSF via bulk flow along the outside of the trigeminal nerve (Thorne, Pronk, Padmanabhan, & Frey, 2004) and thereby bypass the peripheral circulation.

Insulin apart appears to be superior to regular insulin in provoking memory enhancement in AD. (Benedict, et al., 2007) The B28 proline residue in regular insulin is replaced with aspartic acid, inhibiting self-aggregation. Thus, the smaller effective molecular size of insulin aspart appears to allow more efficient CSF access when delivered via nasal spray.

Potential Mechanisms of Insulin's Effect on Cognitive Performance

Neuronal insulin signaling at the hippocampus promotes the phosphorylation and mobilization of intracellular stores of AMPA receptors, increasing their surface expression on post-synaptic membranes. (Ahmadian, et al., 2004; Man, et al., 2000; Trudeau, Gagnon, & Massicotte, 2004) Insulin signaling also promotes phosphorylation and increased activity at NMDA receptors. (Christie, Wenthold, & Monaghan, 1999) Thus, insulin might act to modulate signaling mechanisms known to be central to memory function specifically at the hippocampus.

Insulin might also act in less specific ways to influence cognitive performance. In contrast to its role in skeletal muscle and liver, insulin does not appear to be required for neuronal glucose uptake. Insulin signaling however does promote oxidative metabolism by leading to the phosphorylation and

activation of hexokinase and phosphofructokinase, committing intracellular glucose to the TCA cycle and ATP production. (Hoyer, 1993, 2004a, 2004b) Increased CSF norepinephrine levels correlating with cognitive performance have also been noted after insulin administration in AD, (G. Watson, et al., 2006) suggesting that insulin's effect on memory might involve noradrenergic mechanisms. Noradrenergic signaling in cortex leads to inactivation of slow acting, Ca++ dependent K+ channels, inhibiting after-hyperpolarization and increasing cortical excitability. Insulin is furthermore known to act as a vasodilator peripherally, (E. A. Anderson & Mark, 1993) and augmented delivery of glucose and oxygen via increased tissue perfusion might enhance neuronal function and translate to improved cognitive performance. Thus several physiologic mechanisms, both hippocampal-specific as well as those more general, might contribute to insulin's effect on cognitive performance.

The memory effects of insulin have also been shown to differ according to apolipoprotein-E-epsilon-4 genotype in AD. Cognitive enhancement appears to be most evident in AD non-carriers, (Craft, et al., 2003; S Craft, et al., 2000) and at higher insulin doses (40IU vs 20IU via nasal spray) carriers may show reduced performance. (M. A. Reger, et al., 2006) The apolipoprotein-E-epsilon-4 is a known AD risk factor (Roses & Saunders, 1994) present in up to 60% of AD patients.

(Saunders, et al., 1993) Those AD patients without an apolipoprotein-E-epsilon-4 allele are at increased type 2 diabetes risk compared to AD carriers, (Profenno & Faraone, 2008) suggesting a relationship to peripheral insulin sensitivity, and apolipoprotein-E-epsilon-4 based differences in insulin action although the underlying mechanism is unknown.

The Neural Correlates of Insulin-Mediated Cognitive Enhancement

There is increasing interest in the etiology and novel treatments of AD, and growing body of research suggests that insulin signaling mechanisms might play an important and underappreciated role in both neuronal health and function in this increasingly prevalent disease. Neuronal insulin receptors are found in particularly high density in the hippocampus, and it seems likely that any effects of insulin on brain structure and function would be most evident here. The overall goal of this project was to study insulin's influences on brain structure and function in early AD. A preliminary voxel-based exploration describes the relationship between peripheral insulin and MRI-derived regional brain volume in early AD. Functional MRI was used to assess insulin-related changes in function at the hippocampus and closely related temporal lobe structures during a memory encoding task. Insulin-related changes in brain activation during a simple motor task were assessed as a non-memory control

condition. Arterial spin-labeled MRI was used to measure insulin-related changes in mean cerebral blood. Last, insulin-related changes in cognitive performance were assessed. Considering reports of different cognitive responses to insulin across apolipoprotein E-epsilon-4 genotype in AD, the influence of this known AD risk factor on insulin's effects on brain activity was also examined. The following four specific aims guided our exploration:

Aim 1: Assess the Effects of Insulin on Explicit/Declarative Memory Processes in AD By Comparing Medial Temporal Lobe And Hippocampal Responses to Novel Stimuli with Functional MRI. In addition to being a target of AD pathology, the hippocampus is known to express insulin receptors that might modulate memory performance. Considering the role the hippocampus and related medial temporal lobe structures play in explicit memory function, it seems possible that insulin-related changes in memory performance might be reflected by increased activity at the medial temporal lobe particularly at the hippocampus. It was hypothesized that AD subjects would exhibit increased medial temporal lobe and hippocampal activation to novel stimuli with insulin compared to saline. It was further hypothesized that non-demented subjects would demonstrate no change in activation to novel stimuli with insulin compared to saline.

Summary of Results: It was found that neither the control group nor the overall AD group demonstrated any significant change in hippocampal or related medial temporal lobe activation. The overall AD group did however demonstrate differences in activation between the saline and insulin conditions at several regions outside of the medial temporal lobe that might be linked to memory function.

Aim 2: Determine the Role of Apolipoprotein E-epsilon-4 Genotype in Brain Insulin Responses. Insulin-related memory enhancement appears to exhibit a dose-dependent interaction across apolipoprotein E-epsilon-4 genotype in AD. Non-carrier AD subjects exhibit memory improvement at both 20IU and 40IU doses of insulin administered via nasal spray, while carriers demonstrate attenuated or impaired cognitive performance at the higher 40IU dose. (M. Reger, et al., 2006) It was therefore hypothesized that changes in hippocampal activation during a memory encoding task after a single 40IU intranasal dose of insulin compared to saline would be greater in apolipoprotein E-epsilon-4 negative AD subjects.

Summary of Results: It was found that there was an interaction in insulin related changes in hippocampal activation across apolipoprotein E-epsilon-4 genotype, and that the apolipoprotein E-epsilon-4 positive AD subjects exhibited decreased hippocampal activation with insulin compared to saline, predominantly on the left. The whole brain analysis demonstrated that this apolipoprotein E-epsilon-4 based

interaction also involved the adjacent left parahippocampal gyrus.

Aim 3: Characterize Non-Memory Related Changes in Functional MRI Activation in Response to Insulin. It is not known if insulin might modulate specific anatomy known to possess insulin receptors, such as the hippocampus, or if other regions and structures might exhibit insulin-related changes in function. To characterize insulin-related changes in regions unrelated to memory processes, a simple fMRI motor task was performed during the same scanning session as the memory encoding task. It was hypothesized that motor activity would be greater with insulin compared to saline, but that no differences in this effect would be evident between the diagnosis groups. Insulin is also known to act as a vasodilator peripherally, (E. A. Anderson & Mark, 1993) so mean cerebral blood flow was assessed using arterial spin-labeled MRI. It was hypothesized that mean cerebral blood flow would increase with insulin, without regard for diagnosis.

Summary of Results: It was found that right (ipsilateral) somatosensory cortex demonstrated greater insulin-related activation in the apolipoprotein E-epsilon-4 negative AD group compared to AD carriers, while no insulin-related change in motor cortex activity was demonstrated in the overall AD group. Mean cerebral blood flow as measured with ASL-MRI was the same in both the saline and insulin

conditions in both diagnosis groups as well as the overall group.

Aim 4: Determine Insulin's Effects on Cognitive Performance. Previous reports indicate that AD subjects, particularly those who do not carry an apolipoprotein E-epsilon-4 allele, demonstrate improved memory performance after insulin administration. A brief image recall test and battery of standardized cognitive tests were performed to serve as a validation of the methods employed. It was hypothesized that AD subjects, particularly those without an apolipoprotein E-epsilon-4 allele, would show improved memory performance with insulin compared to saline.

Summary of Results: It was found that apolipoprotein E-epsilon-4 negative AD subjects demonstrated greater image recall performance with insulin compared to saline, while no change in performance was evident in the apolipoprotein E-epsilon-4 positive AD group, or in the overall AD or control groups. No difference in performance on the standardized cognitive tests between the saline and insulin conditions was evident in the control or overall AD group, or in either apolipoprotein E-epsilon-4 subgroup.

In sum, the work presented herein will lead to submission of three unique manuscripts for publication. 1) *Hippocampal and Limbic Volume Are Related to Insulin Levels in Early Alzheimer's Disease*, 2) *Insulin Modulates Hippocampal Activation During Memory Encoding in Early AD According to Apolipoprotein E-epsilon-4 Genotype*, and *Insulin and fMRI Motor Activity in Early Alzheimer's Disease*.

Chapter 2-Hippocampal and Limbic Volume Are Related to Insulin Levels in Early Alzheimer's Disease

Hippocampal and Limbic Volume Are Related to Insulin Levels in Early Alzheimer's Disease

Abstract

Insulin signaling appears to influence the synthesis of both beta-amyloid and neurofibrillary tangles in Alzheimer's disease, and therefore might act as an important modulator of Alzheimer's neuropathology. Our earlier studies have demonstrated a positive correlation between peripheral insulin and whole brain volume in AD, suggesting that higher insulin may be neuroprotective. We examined the relationship between peripheral insulin during a 3-hour intravenous glucose tolerance test and regional brain volume in early Alzheimer's disease using voxel-based morphometry, hypothesizing that insulin levels would correlate with volume in regions known to express insulin receptors, specifically at the hippocampus. Our results demonstrate a positive correlation between insulin and hippocampal volume in AD, but not controls. We further found a positive correlation between insulin and limbic/paralimbic gray and white matter volume. Lastly, insulin positively correlated with hypothalamic volume in apolipoprotein E-epsilon-4 negative AD subjects, but not in AD subjects with the gene. Our results suggest that insulin might act as an important neurotrophic factor in AD, and add to the growing body of evidence linking insulin signaling and AD.

Background

Insulin's role in peripheral glucose metabolism is well known, but a growing body of research suggests impaired insulin signaling may be associated with Alzheimer's disease (AD). Type 2 diabetes mellitus is a risk factor for developing AD (Peila, Rodriguez, Launer, & Honolulu-Asia Aging, 2002) and higher peripheral glucose and insulin levels are reported in early AD compared to control subjects (Craft, et al., 1998) suggesting that altered insulin action might be a component of AD pathophysiology. The majority of brain insulin is synthesized in the pancreas and traverses the blood-brain barrier to reach the central nervous system. (Banks, 2004) A recent report indicates that impaired insulin secretion during mid-life may be a risk factor for AD in later life, (Ronnemaa, et al., 2008) further associating alterations in peripheral insulin signaling with AD risk.

Impaired insulin action within the brain might increase AD risk by contributing to beta-amyloid and tau deposition. Mechanistically, brain insulin signaling inhibits amyloid plaque formation by modulating the transport and processing of amyloid fragments through the Golgi apparatus through its influence on glycogen synthase kinase 3a. (Phiel, et al., 2003) Insulin signaling also inhibits tau hyperphosphorylation, (M Schubert, et al., 2003) a key step in the formation of neurofibrillary tangles found in AD. Autopsy studies have demonstrated reduced

brain insulin receptors as well as their respective signaling intermediates in AD (Steen, et al., 2005) providing evidence at the molecular level of altered brain insulin signaling in AD. It appears therefore that faulty insulin signaling mechanisms in the brain might be associated with AD. Several reports suggest that augmentation of insulin action might modulate AD neuropathology, further linking insulin and AD neuropathology. Post-mortem examination of brains of diabetics with a history of using both insulin and other diabetes medications showed fewer neuritic plaques specifically in the hippocampus, amygdala and entorhinal cortex, suggesting that increased insulin effects might ameliorate or retard the formation of the neuropathologic hallmarks of AD. (Beeri, et al., 2008) Furthermore, increased plasma amyloid beta 40/42 ratio has been described after extended insulin treatment in AD, (S Craft, et al., 2000) suggesting that insulin might shift amyloid metabolism toward production of the less amyloidogenic Abeta40 species. Higher insulin levels however have also been speculated to contribute to AD neuropathology. Natural substrates of the metalloproteinase insulin degrading enzyme (IDE) include both insulin and amyloid beta. (James Scott Miners, 2008) Higher insulin levels therefore might compete with amyloid beta for degradation by IDE (I. V. Kurochkin, 2001) promoting amyloid accumulation.

Mounting evidence therefore suggests that insulin may serve as a modulator of both beta amyloid plaques and neurofibrillary tangles characteristic of AD, and that altered brain insulin action might promote AD neuropathology.

The apolipoprotein E-epsilon-4 allele is a known AD risk factor, (Roses, 1994, 1998; Roses & Saunders, 1994) that also appears to be associated with altered insulin metabolism in AD.(S Craft, S Asthana, G Schellenberg, et al., 1999) Cognitive changes in response to experimental insulin administration in AD (S Craft, S Asthana, JW Newcomer, et al., 1999; S. Craft, et al., 2000; Craft, et al., 1996; M. Reger, Watson, Green, Wilkinson, et al., 2008; G. Watson, et al., 2006) exhibit an interaction with the apolipoprotein E-epsilon-4 allele, with non-carriers showing performance improvements in list recall after a single 40IU dose of insulin delivered intranasally, whereas APOE–e4 positive AD subjects exhibit reduced cognitive performance at the same insulin dose. (M. Reger, et al., 2006) This effect appears to be AD-specific as normal participants did not exhibit the cognitive changes seen in the demented subjects. The apolipoprotein E-epsilon-4 allele may therefore be an important factor in modifying insulin's putative effect on brain health and physiology in AD. The biochemical mechanisms underlying these observations are not delineated, but APOE-e4 genotype might be expected to influence any neurotrophic effects of insulin.

Neuroimaging is increasingly used to quantify whole brain, limbic and hippocampal volume loss (Callen, Black, Gao, Caldwell, & Szalai, 2001; Jack, et al., 2002; Sluimer, et al., 2008) as a reflection of pathologic burden. (Borthakur, Sochor, Davatzikos, Trojanowski, & Clark, 2008; Clark, et al., 2008; Hall, Moore, Lopez, Kuller, & Becker, 2008; Zhou, et al., 2008) Neuronal insulin receptors are not uniformly distributed, but rather found in high concentrations in selective regions including the hippocampus and hypothalamus, where they may function to modulate neuronal survival and AD neuropathology. (Frolich, et al., 1998; Frolich, et al., 1999; Hoyer, 2004a; Schulingkamp, Pagano, Hung, & Raffa, 2000)

Earlier we described a positive correlation between whole brain volume obtained from MRI data and peripheral insulin levels during a 3-hour I.V. glucose tolerance test in early AD. (Burns, et al., 2007) These findings suggest that insulin might influence brain structure, perhaps by modulating AD neuropathology. Our objective for the current study was to extend our earlier findings by determining the regional specificity of the peripheral insulin and whole brain volume relationship in AD (Burns, et al., 2007) by using a voxel-wise regional analysis. We hypothesized distinct differences in the relationship between insulin and hippocampal volume across the control and AD groups, and that insulin levels would positively correlate with hippocampal gray matter volume specifically in AD subjects. We also explored the relationship between insulin and whole-brain gray and white matter volume on a

voxel-wise basis. Lastly, apolipoprotein E-epsilon-4-based differences in the relationship between insulin and whole brain structure were examined.

Methods

Participants: Data from 138 (70 AD and 68 non-demented) University of Kansas Brain Aging Program volunteers (Burns, Cronk, et al., 2008; Burns, et al., 2007; Burns, Mayo, Anderson, Smith, & Donnelly, 2008) were initially reviewed to identify those with complete IVGTT data. MRI data were examined visually and excluded if excessive motion artifacts or other technical defects that might contribute to normalization or segmentation inaccuracies were present. A total of 59 early AD patients (61% females) and 55 non-demented controls (58% females) with complete IVGTT and acceptable MRI data were identified. All participants provided informed consent in accordance with the University of Kansas Medical Center Human Subjects Committee. Subjects underwent a thorough exam, including an interview with a collateral source familiar with the subject to establish the presence of Alzheimer's disease. Diagnosis was based on gradual and progressive memory impairment and deficit in at least one other cognitive domain, (L Berg, et al., 1988) with particular attention to intrasubject change, rather than deviation from group norms. (Morris, 1997) All demented participants met the NINDS-ADRDA criteria for probable Alzheimer's disease. Disease severity was graded with the Clinical Dementia Rating (CDR) scale. (Morris, 1993) Participants receiving a CDR score of 0.5 (n=46) or 1 (n=13) were included as AD subjects, whereas those scoring 0 served as controls. Exclusion criteria included diabetes (defined as insulin or oral hypoglycemic agent use by self-report or fasting blood glucose > 126 mg/dl),

disorders other than AD with the potential to impair cognition, stroke, clinically significant depressive symptoms, abnormalities in vitamin B12 levels, positive rapid plasma reagin, abnormal thyroid function, or the concurrent use of psychoactive or investigational medications.

Insulin Measurement: A three-hour intravenous glucose tolerance test (IVGTT) was performed at 8:30 AM after a 12-hour fast. Blood samples for determination of glucose and insulin levels were collected 5 minutes before and at 1, 3, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, and 180 minutes after delivery of a glucose bolus (0.3g/kg body weight). (Burns, et al., 2007) The integrated insulin response to the glucose challenge was calculated using the trapezoid method and reported as insulin area-under-the-curve (iAUC). Although the insulin AUC measurement used in this study represents endogenous insulin response to an experimental glucose challenge, higher values are associated with higher fasting insulin levels as well as increased postprandial endogenous insulin production on a day-to-day basis. Insulin AUC during the 3-hour IVGTT therefore serves as an index of chronic insulin exposure.

MRI Acquisition and Image Processing: T1-weighted MRI data (magnetization-prepared rapid gradient echo (MPRAGE); TR=2500ms, TE=4.38ms, TI=1100ms, flip angle=8 degrees, field of view 256x256mm^2 with 18% oversample, 1-mm slice thickness, $1mm^3$ voxels) were acquired with a 3T Siemens Allegra scanner (Siemens, Erlangen, Germany).

Statistical Analysis: Structural MRI data were segmented into gray and white matter maps using the VBM5 toolbox (http://dbm.neuro.uni-jena.de), an extension of SPM5 (Statistical Parametric Mapping, Wellcome Department of Cognitive Neurology, London, UK). The final tissue maps were modulated in order to analyze regional volumes, and smoothed with a 10mm FWHM Gaussian kernel. Regression analysis of the control and AD subgroups separately was used to test the hypothesis that insulin was correlated with hippocampal volume, limiting the search volume to the left and right hippocampi using the Wake Forest University Pick atlas tool (Maldjian, Laurienti, Kraft, & Burdette, 2003). Each participant's gray matter data was specified as the dependent variable, with insulin, age and sex entered as regressors into the model. Statistical parametric maps were generated demonstrating the relationship between insulin and hippocampal gray matter volume, controlling for age and sex. Differences in the relationship between insulin and hippocampal gray matter voles were assessed by specifying a factor (diagnosis) with two levels (control and AD), again controllong for age and sex. For these hypothesis-driven

analyses, voxels correlating with insulin with a p-value < 0.05 corrected for multiple comparisons (false discovery rate, FDR) were accepted as significant.

Whole brain statistical parametric maps were generated for the subsequent within-groups exploratory analysis, demonstrating the relationship between insulin and gray and white matter volume, while controlling for age and sex. Any voxels correlating with insulin, with a more liberal uncorrected default p-value < 0.001, were considered significant for these analyses. Interaction analysis was performed to explore group differences in the relationship between insulin and regional gray and white matter volume. The gray and white matter data were again specified as dependent variables, with insulin, age and sex selected as regressors. A factor (i.e. diagnosis group) with two levels (AD or control) was created to model interactions between dementia status and insulin. Statistical parametric maps were generated to demonstrate differential relationships between insulin and brain volume across groups controlling for age and sex. Any voxels exhibiting a between-group interaction with an uncorrected p-value <0.001 were considered significant.

Finally, interaction analysis was used to assess apolipoprotein E-epsilon-4-based differences in the relationship between insulin and brain structure within the AD group. A two level factor (carrier or non-carrier, without regard for allelic load) was created to model interactions between genotype and insulin. Statistical parametric maps were generated to demonstrate differential relationships between insulin and brain volume across groups controlling for age and sex. Any voxels exhibiting a between-group interaction with an uncorrected p-value <0.001 were considered significant.

Demographic characteristics of the subjects (Table 1) were analyzed with SPSS 16.0 (SPSS, Chicago, USA) using the student's t-test and expressed as mean ± SD. Chi^2 was used to test for equality of proportions. All images are displayed at an uncorrected p-value of 0.01, and a minimum cluster size of 100 voxels. The peaks of the imaging analyses are reported in Table 2 by Talairach (x, y, z) coordinates, along with the computed Z-scores and cluster sizes.

Sample Characteristics: The mean age of participants was 73.7 years (SD 6.4), with no difference between AD or control groups. (Table 2-1) There were no group differences in sex distribution (chi square=0.095, df=1, p=0.76), or 3-hour insulin AUC (t=1.38, p=0.17). There was a trend towards more education in the

control group. (16.5±2.3y vs. 15.3±3.4y for the AD group, t=1.908, p=0.06) Apolipoprotein E-epsilon-4 genotype was available for 56 of the AD subjects (26 apolipoprotein E-epsilon-4 negative and 30 apolipoprotein E-epsilon-4 positive).

Insulin and Hippocampal Volume: The small volume corrected analysis demonstrated a positive correlation between insulin and hippocampal gray matter volume in the AD subjects on both the left and right, most prominently at the posterior right (18, -3, -12; Z = 3.24, p=0.05, FDR) hippocampus. The interaction analysis demonstrated a trend towards group-wise differences in the insulin-hippocampal volume relationship at the posterior aspect of the right hippocampus. (36, -37, -4, Z=3.37, p<0.001 uncorrected, p=0.12 FDR-corrected) (Figure 2-1) There were no regional correlations between insulin and hippocampal gray matter volume in the control group.

Insulin and Regional Gray Matter Volume: Exploratory whole-brain analysis of the AD group demonstrated a positive correlation between insulin and limbic gray matter volume including the cingulate gyri, both insulae and the right medial temporal lobe, with a global peak at the left insula (-42, 3, 0; Z = 4.11, p<0.001 uncorrected) (Figure 2-2). In controls, a positive correlation between insulin and gray matter was identified at a few scattered foci in the right occipital and left frontal lobes, with the greatest correlation in the right occipital region (41, -84, 3; Z = 3.46, p<0.001 uncorrected). Interaction analysis confirmed group differences in the

correlation between insulin and gray matter volume, most prominently at the right parahippocampal gyrus (37, -51, -6; $Z = 4.19$, $p<0.001$ uncorrected) (Figure 2-2) where a positive correlation was present in the AD group but not in the controls. A cluster of 541 voxels located within the right pre-motor region (44, 1, 51; $Z = 3.86$, $p<0.001$ uncorrected) demonstrated a negative correlation with insulin in the control group (not shown). Voxels with positive correlations between insulin and gray matter volume (p-values< 0.001, uncorrected for multiple comparisons) are listed in Table 2-2.

Insulin and Regional White Matter Volume: Results of exploratory analysis of the insulin-white matter volume relationship paralleled the insulin-gray matter volume relationships. The control group showed several small, isolated clusters in the right posterior temporal lobe region that correlated with insulin, most prominently at the right occipital lobe (29, -56, -15; $Z = 3.51$, $p<0.001$ uncorrected). The AD group however showed an extensive positive correlation between insulin and paralimbic white matter, most prominently at the right posterior temporal lobe (36, -52, 3; $Z = 3.98$, $p<0.001$ uncorrected) (Figure 2-3). Interaction analysis confirmed group differences in the correlation between insulin and white matter volume, most prominently near the right cuneus (19, -75, 13; $Z = 3.97$, $p<0.001$ uncorrected) (Figure 2-3), where a positive correlation was present in the AD group compared to the controls. No negative correlations between white matter volume and insulin were present. Voxels with positive correlations between insulin and white matter volume (p-values < 0.001, uncorrected for multiple comparisons) are listed in Table 2-2.

Apolipoprotein E-epsilon-4-based differences in the insulin-brain structure relationship: We first assessed apolipoprotein E-epsilon-4-based differences in the relationship between insulin and gray matter volume, again restricting the search volume to the hippocampus with the Wake Forest University Pick atlas. This analysis revealed no apolipoprotein E-epsilon-4 group differences in this relationship

at the hippocampus. Voxel-wise while brain analysis of apolipoprotein E-epsilon-4-based differences in the relationship between insulin and gray matter however revealed an interaction at two locations (8, -1, -3 and -7, -3, -5; $p<0.001$ uncorrected) where a positive correlation was present in the apolipoprotein E-epsilon-4 negative AD but not in the apolipoprotein E-epsilon-4 positive AD group. (Figure 2-4) The corresponding Talairach coordinates map to the hypothalamus. Comparing the apolipoprotein E-epsilon-4 positive to the apolipoprotein E-epsilon-4 negative AD group revealed no voxels where the insulin-gray matter correlation was greater in the apolipoprotein E-epsilon-4 positive AD group compared to the apolipoprotein E-epsilon-4 negative AD group.

Discussion

We reported earlier that peripheral insulin positively correlated with whole brain volume in early AD. (Burns, et al., 2007) The current study extends our earlier findings by demonstrating an AD-specific relationship between insulin and hippocampal volume. Our exploratory analysis of regional gray and white matter furthermore demonstrates that higher peripheral insulin levels are associated with higher gray and white matter volume in limbic and paralimbic structures, regions that bear significant pathologic burden in AD. Although the specific mechanism underlying our observations cannot be determined from this study, our results are consistent with other studies suggesting attenuation of AD neuropathologic burden in those brain areas known to express insulin receptors and to bear a large pathologic burden in AD (Beeri, et al., 2008; S. Craft, et al., 2000; Phiel, et al., 2003; M Schubert, et al., 2003) Alternatively, insulin might act specifically on insulin-sensitive limbic anatomy to exert neurotrophic effects. (Craft & Watson, 2004; Francis, et al., 2008; Gasparini & Xu, 2003; M. Schubert, et al., 2004)

Our findings may also represent attenuation of atrophy rates in insulin-sensitive regions in subjects with higher insulin levels. In sum, these findings suggest that higher insulin exposure in AD might modulate the progression of neuropathology, although longitudinal studies would be required to substantiate this

argument.

Although we did not find Apo-e4 related differences in the relationship between insulin and hippocampal volume, our whole-brain analysis identified an interaction across apolipoprotein E-epsilon-4 groups in the hypothalamus, with a positive relationship between insulin and hypothalamic gray matter volume in apolipoprotein E-epsilon-4 negative AD subjects that was not present in the apolipoprotein E-epsilon-4 positive group. This is particularly interesting given the central role of the hypothalamus in endocrine control including regulating insulin secretion. (Bobbioni-Harsch & Jeanrenaud, 1990) It is not clear from this cross sectional study if higher insulin levels contribute to preserved hypothalamic volume in apolipoprotein E-epsilon-4 negative AD, or if greater hypothalamic volume is associated with greater insulin response to the glucose challenge in these subjects. These findings suggest that insulin might exert beneficial neurotrophic effects at the hypothalamus in apolipoprotein E-epsilon-4 negative AD, and add to the growing body of evidence that the apolipoprotein E-epsilon-4 allele is an important consideration when assessing insulin in AD.

Since the cross-sectional design limits the inferences that can be made from this study, our findings need confirmation in longitudinal and interventional trials to establish if insulin influences regional brain atrophy rates in AD. Although we used sensitive and validated clinical methods to identify subjects with the earliest stage of Alzheimer's disease, (Morris, 1997) postmortem examination is the most accurate method of diagnosis. The findings in the white matter of AD patients in particular should be interpreted with caution as these might be related to periventricular white matter changes that have the potential for misclassification in VBM. (Levy-Cooperman, Ramirez, Lobaugh, & Black, 2008) Although the optimized method we used reduces such error, (Good, et al., 2001) systematic misclassification of white matter lesions as gray matter in the AD group might lead to over-modulation of adjacent white matter regions, generating fictitious regional white matter atrophy in AD. Lastly, we used peripheral measures of insulin as a surrogate measure for CNS insulin. Although insulin within the CNS is derived primarily from the periphery, (Banks, 2004) peripheral values may not accurately reflect CNS insulin levels as the blood-brain barrier transport of insulin may be altered in AD. (Banks, 2004)

Table 2-1. Sample Characteristics

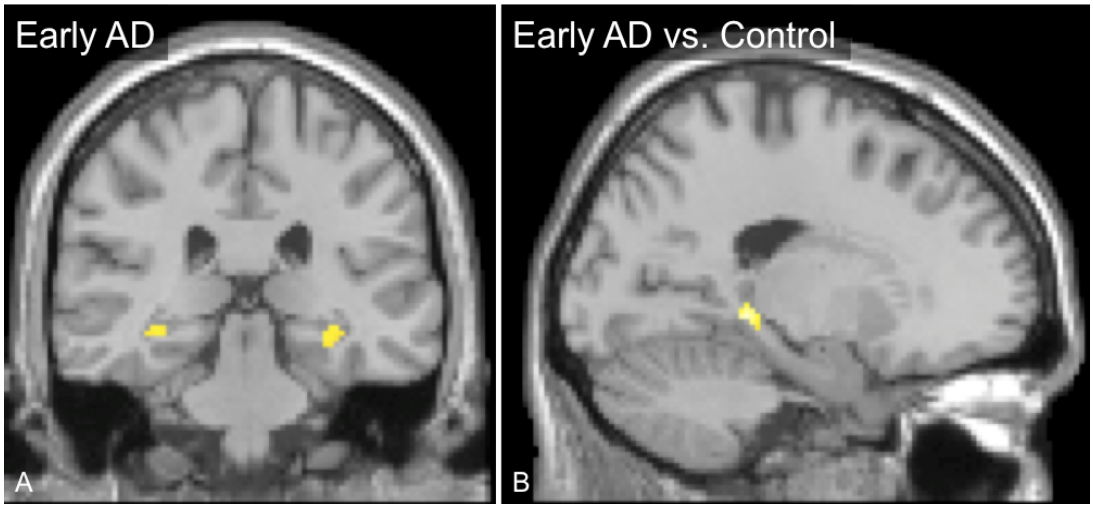
	Control (n=55) Mean (SD)	AD (n=59) Mean (SD)	p-value
Age (years)	73.13 (6.19)	74.29 (6.55)	0.33
Education (years)	16.35 (2.29)	15.32 (3.37)	0.06
MMSE	29.44 (0.76)	26.27 (3.64)	<0.001
Insulin AUC (uU*ml/min)	2459 (1188)	2857 (1850)	0.17

Table 2-2. Summary of Suprathreshold Peaks. Brodmann areas and location names were determined from the Talairach Client after converting the MNI coordinates reported by SPM to Talairach coordinates.

Group	Tissue Class	Brodmann Area/Location	MNI Coordinates (x, y, z)	z-score	Cluster Size (voxels)
Control	Gray Matter	19	41, -84, 3	3.46	316
		9	-22, 27, 37	3.40	111
	White Matter	Right Precuneus	18, -64, 22	3.39	106
AD	Gray Matter	13	-42, 3 0 4.11	4.11	2579
		6	-27, 4, 47	3.61	168
		21	-62, -4, -13	3.56	161
		Left LFN	-10, 1, 5	3.78	103
		31	3, -44, 29	3.43	400
		21	-68, -30, -1	3.38	101
		13	40, 5, -2	3.38	121
		19	42, -74, -6	3.38	115
		29	-4, -57, 11	3.33	140
	White Matter	Right Temporal Lobe	36, -52, 3	3.98	2328
		Left PHG	-20, -35, -9	3.83	1252
			19, -52, 13	3.74	572
		Right CG	14, -20, 36	3.70	632
		Left Frontal Lobe	-39, 23, 3	3.69	117
		Left Frontal Lobe	-25, -34, 21	3.59	288

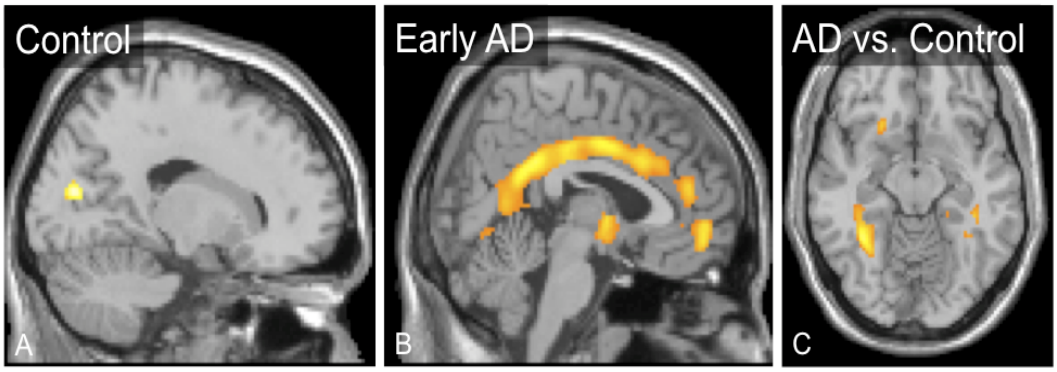
PHG-parahippocampal gyrus, CG-cingulate gyrus, LFN-lentiform nucleus

Figure 2-1-Peripheral Insulin and Hippocampal Gray Matter Volume.



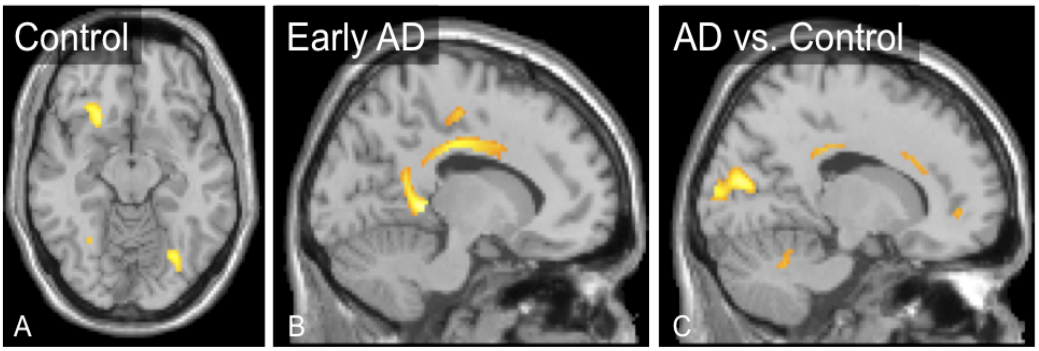
Peripheral insulin during a 3-hour IVGTT correlated with gray matter volume at the hippocampus in AD (A, p=0.05 FDR) but not in controls. Interaction analysis (B) confirmed group differences in the relationship between insulin and hippocampal volume. The yellow cluster in B denotes where the positive correlation between insulin and hippocampal volume in AD was significantly different from controls. (p=0.09 FDR, p<0.001 uncorrected) The search volume was restricted to the hippocampus for these analyses.

Figure 2-2-Regional Relationships Between Insulin and Gray matter Volume.



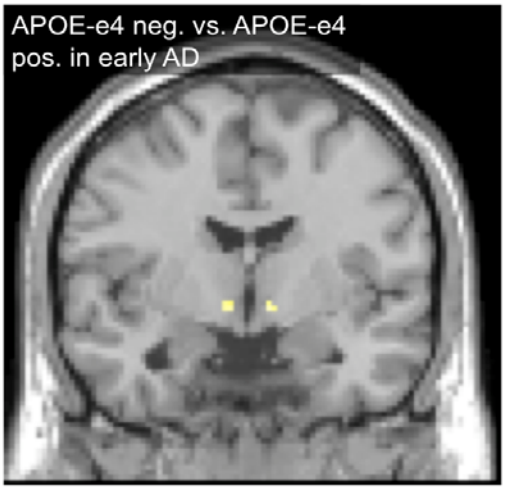
Control subjects demonstrated a relationship in several scattered regions including the occipital lobe (A), while an extensive limbic/paralimbic relationship was present in the AD group. Interaction analysis confirmed these group-wise differences, most significantly at the right parahippocampal region (C) These whole-brain analyses were thresholded at $p<0.001$ uncorrected for multiple comparisons.

Figure 2-3-Regional Relationships Between Insulin and White Matter Volume.



Control subjects demonstrated a relationship in several scattered regions including the frontal and occipital lobes. (A) Similar to the gray matter findings, the AD group showed an extensive limbic/paralimbic relationship with insulin. (B) Interaction analysis confirmed these group-wise differences. (C) These whole-brain analyses were thresholded at $p<0.001$ uncorrected for multiple comparisons.

Figure 2-4-APOE-e4-Related Differences in The Relationship Between Insulin and Regional Gray Matter.



There was a significant difference in the relationship between insulin and hypothalamic gray matter volume across APOE-e4 genotype, with greater insulin levels associated with greater hypothalamic volume in APOE-e4 negative AD. This whole-brain analyses was thresholded at $p<0.001$ uncorrected for multiple comparisons.

Chapter 3-Insulin Modulates Hippocampal Activation During Memory Encoding in Early AD According to Apolipoprotein E-epsilon-4 Genotype

Insulin Modulates Hippocampal Activation During Memory Encoding in Early AD According to Apolipoprotein E-epsilon-4 Genotype

Abstract

Several descriptions of insulin-related memory improvement in early Alzheimer's disease subjects suggest that this hormone might modulate brain function. Insulin's effect on memory furthermore appears to differ according to apolipoprotein E-epsilon-4 genotype in AD with greater memory improvement in non-carriers. The neural substrates of this phenomenon are not clear, however the hippocampus is known to express insulin receptors that might influence memory processes. Modulation of hippocampal function by insulin might therefore be involved in insulin-related memory facilitation in Alzheimer's disease. The goal of this study was to characterize changes in hippocampal activation after insulin administration using functional MRI during a memory encoding task. It was hypothesized that hippocampal activation would be greater during the memory encoding task with insulin compared to saline in Alzheimer's disease subjects, particularly those who did not possess an apolipoprotein E-epsilon-4 allele.

The results demonstrated an AD-specific interaction in hippocampal activation between the saline and insulin conditions across apolipoprotein E-epsilon-4 genotype. The apolipoprotein E-epsilon-4 negative AD subjects exhibited increased image recall performance with insulin compared to saline, which positively correlated with insulin-related changes in hippocampal percent activation volume. Image recall performance in demented carriers of the apolipoprotein E-epsilon-4 allele was similar between the saline and insulin conditions, but this group showed a significant decrease in hippocampal percent activation volume after insulin administration.

These results suggest that insulin might act at the hippocampus in apolipoprotein E-epsilon-4 positive AD subjects to promote cognitive function described herein and by others. The observation of insulin-related decrease in hippocampal percent activation volume along with constant image recall performance seen in the apolipoprotein E-epsilon-4 positive AD group might indicate increased hippocampal efficiency in this subgroup. The interaction in hippocampal percent activation volume across apolipoprotein E-epsilon-4 genotype in AD subjects suggests that this allele defines two AD subpopulations with fundamentally different neuromodulatory insulin responses.

Background

Converging evidence implicates defective insulin signaling in Alzheimer's disease (AD) pathogenesis (Benedict, et al., 2007; Craft, 2006; S Craft, et al., 2000; Craft, et al., 1998; Hoyer, 2004a, 2004b; Jones & Leonard, 2005; Kim, et al., 2006; Messier & Teutenberg, 2005a; van der Heide, Hoekman, Biessels, & Gispen, 2003). Altered insulin signaling for example has been shown to contribute to AD neuropathology. (Hoyer, 2004a, 2004b; Kim, et al., 2006; van der Heide, et al., 2003) Non-diabetic AD patients also may exhibit higher peripheral glucose and insulin levels, a profile similar to that seen in type 2 diabetes. (Craft, et al., 1998) A recent report indicates that impaired insulin secretion during mid-life may be a risk factor for AD in later life, (Ronnemaa, et al., 2008) and type 2 diabetics are at increased AD risk, (Peila, et al., 2002) further suggesting an association between insulin dysregulation and AD.

The role of insulin in brain health and function is currently not fully delineated, but insulin may have a modulatory role in memory function. For example, injection of insulin into the cerebral ventricles of rats improves memory. (Park, Seeley, Craft, & Woods, 2000) Furthermore, intraventricular injection of streptozotocin, which interferes with insulin signaling mechanisms, leads to cognitive impairment in rats. (Lannert & Hoyer, 1998) Insulin signaling appears to

be linked to memory function at the molecular level. The majority of brain insulin is formed in the pancreas and traverses the blood-brain barrier to reach the central nervous system, (Banks, 2004) where insulin receptors are found in high density in specific regions including the olfactory bulbs, hypothalamus and the hippocampus. Neuronal insulin signaling at the hippocampus promotes the phosphorylation and mobilization of intracellular stores of AMPA receptors, increasing their surface expression on post-synaptic membranes. (Ahmadian, et al., 2004; Man, et al., 2000; Trudeau, et al., 2004) Insulin signaling also promotes phosphorylation and increased activity at NMDA receptors. (Christie, et al., 1999) Thus, neuronal insulin signaling might be linked to cognitive function via modulation of long-term depression (LTD) and long term potentiation (LTP) mechanisms specifically at the hippocampus.

Insulin's potential role in cognitive function, particularly in AD, is supported by several reports of memory improvement after exogenous insulin administration. Intravenous injection of insulin in AD subjects, along with dextrose to maintain glucose at baseline levels, increases CSF insulin levels and acutely improves performance on cognitive tasks such as list and word recall. (S Craft, S Asthana, JW Newcomer, et al., 1999; Craft, et al., 1996) Insulin administration via nasal spray has been shown to promote cognitive performance in AD similar to IV administration, without hypoglycemia risk. (M. Reger & S. Craft, 2006; M. Reger, et al., 2006; M. Reger, Watson, Green, Baker, et al., 2008; M. Reger, Watson, Green,

Wilkinson, et al., 2008) Insulin administered in this manner appears to travel along the outside of trigeminal nerves via bulk flow to access the CSF, bypassing the bloodstream. (Thorne, et al., 2004) The memory effects of insulin appear to be most pronounced in APOE-ε4 negative AD subjects, (Bassett, et al., 2006; Craft, et al., 1998; Kukull, et al., 1996; Mosconi, et al., 2004; Pedersen, et al., 2006; M. Reger, et al., 2006; M. A. Reger & S. Craft, 2006; Risner, et al., 2006; G. Watson, et al., 2005) suggesting that the apolipoprotein E-epsilon-4 allele might interact with insulin signaling to modulate memory facilitation.

The anatomic structures mediating insulin's cognitive effects are unknown, however the hippocampus is a likely substrate. In addition to being a target of AD pathology, hippocampal neurons express insulin receptors that might influence memory performance. Insulin treatment has been shown to lead to transient phosphorylation of tyrosine residues of NR2A and NR2B NMDA receptor subunits in rat hippocampus. (Christie, et al., 1999) Insulin-related facilitation of NMDAr activity in xenopus oocytes has also been described. (Liu, Brown, Webster, Morrisett, & Monaghan, 1995) Long-term potentiation was found to be impaired in diabetic rats hippocampus, but this function was restored with insulin treatment. (Izumi, Yamada, Matsukawa, & Zorumski, 2003) Insulin therefore appears to play a role in NMDA activity and hippocampal long-term potentiation mechanisms that might contribute to insulin-mediated cognitive enhancement seen in AD.

The primary goal of this pilot study was to characterize hippocampal responses to insulin in early AD, and secondarily to assess the role of apolipoprotein E-epsilon-4 genotype in modulating hippocampal insulin responses. We hypothesized that early AD subjects would exhibit increased hippocampal activity during a memory encoding task with insulin compared to saline placebo. We furthermore hypothesized that insulin would have a greater effect on hippocampal activity in apolipoprotein E-epsilon-4 negative AD subjects, paralleling reports of differential insulin memory effects along apolipoprotein E-epsilon-4 genotype in AD. We also performed a battery of standardized cognitive tests to replicate earlier reports and link any insulin-related memory changes with imaging findings.

Methods

Subject Selection: All participants were right-handed non-diabetics over the age of 60, and provided written informed consent in accordance with the University of Kansas Medical Center Human Subjects Committee. Diagnostic criteria for AD included gradual and progressive memory impairment and deficit in at least one other cognitive domain. (L. Berg, et al., 1988) A thorough exam, including an interview with a collateral source familiar with the subject, was performed to characterize the presence and severity of dementia with the Clinical Dementia Rating (CDR) scale, (Morris, 1993) focusing on intrasubject change rather than deviation from group norms. (Morris, 1993, 1997; Rockwood, Strang, MacKnight, Downer, & Morris, 2000) Participants included AD subjects with CDR scores of 0.5 and 1, while those with CDR scores of 0 served as controls. Exclusion criteria (Burns, et al., 2007) included diabetes (defined as self-reported insulin or oral hypoglycemic agent use, or fasting blood glucose > 126 mg/dl), disorders other than AD with the potential to impair cognition, stroke, clinically significant depressive symptoms, abnormalities in vitamin B12 levels, positive rapid plasma reagin, abnormal thyroid function, or concurrent use of psychoactive or investigational medications. Apolipoprotein E-epsilon-4 genotype in the AD subjects was determined by allelic discrimination. Participants were not excluded from this pilot study if they were taking AD medications such as cholinesterase inhibitors or NMDA agonists.

All participants were scanned on two occasions at least 48 hours apart in a double-blind, crossover, and counterbalanced manner. All scanning was performed in the morning after an overnight fast. Participants were instructed to take any morning medications as usual, and to maintain their usual caffeine intake on study days. For each visit, either 40IU insulin aspart, (Benedict, et al., 2007) or an equal volume of saline placebo, was administered via nasal spray. (M. A. Reger & S. Craft, 2006) An IV line was placed prior to insulin administration for pre- and post-drug/placebo glucose and insulin testing, and for ready vascular access for dextrose infusion if necessary due to hypoglycemia. Subjects were shown a brief video for familiarization with the fMRI tasks.

Imaging: A Siemens 3T Allegra scanner using a quadrature head coil was used for all imaging. A T1-weighed image (Magnetization-Prepared Rapid Gradient Echo [MPRAGE]; TR=2500 ms, TE=4.38 ms, TI=1100ms, flip angle=8 degrees, field of view 256x256mm with 18% oversample, slice thickness/gap/number=1mm/0/208) was acquired to provide a backdrop on which to overlay functional data, and to provide anatomic information for hippocampal mask creation. Blood-oxygen level dependent (BOLD) sensitive EPI data were acquired parallel to the AC-PC line. (FOV=192x100, slice thickness/gap/number=3.0mm/0.5mm/43, TR=3000, TE=40, FA=90, 198 data points)

Memory Encoding Stimulus: The 10 minute memory encoding stimulus consisted of alternating blocks of novel and repeated image groups, separated by fixation blocks of equal duration. (Golby, et al., 2005) Each block of novel stimuli consisted of 8 unique images (6 outdoor and 2 indoor scenes) presented for 2500ms (with 500 ms interstimulus gap) in a pseudorandom order (total block duration = 24 seconds). To ensure active task engagement, subjects were instructed to respond by button press when presented with an outdoor scene. The phrase "Outdoors?" was placed unobtrusively at the lower margin of each image to remind participants of the task. The repeated stimulus blocks were composed of a single outdoor image displayed 6 times along with a single indoor image, displayed twice in a pseudorandom order with the same timings, durations and response instructions as for the novel stimuli blocks. A white cross on a black background was displayed during the fixation blocks. Subjects were visually cued to press the response button 6 regularly spaced times during the fixation blocks, keeping the number of expected button presses (6) equal across all blocks of the stimulus. The order of novel and repeat block presentation was counterbalanced between sessions, and different image sets were shown for each visit. Stimuli were presented with commercial software (Presentation, NeuroBehavioral Systems) using an LCD back projection system with MRI-compatible vision correction when necessary. Images used for the memory encoding stimulus were obtained from commercially available sources (Corel, Inc.) and selected for similar detail, content, emotional valence and depicted activities.

Region-of-Interest Based Functional MRI Data Analysis: We chose to characterize hippocampal activation in each individual for each session as hippocampal percent activation volume (HPAV) using an anatomically-based region-of-interest (ROI) approach. Since brain atrophy has been shown to influence fMRI activation, (Brodtmann, Puce, Darby, & Donnan, 2009) our goal was to minimize the potentially confounding influence of hippocampal volume variability related to intersubject differences and AD-related atrophy. (Figure 3-1) The first step in HPAV calculation required the creation of left and right hippocampal masks based on the T1 data from each session using established protocols. (Burns, et al., 2007; Jack, et al., 1999) The tracings were done with an 8x6 inch digitizing tablet by a single operator. (GPT) Volumes for the left, right and total hippocampal masks were calculated for each subject/session by multiplying the number of mask voxels by voxel volume.

The functional data was next processed and analyzed with SPM5 (Wellcome Trust Centre for Neuroimaging, University College London). The first 6 seconds of functional data were discarded to allow for image stabilization. The BOLD data underwent slice-timing correction, 3D motion correction and smoothing with a 4mm isotropic Gaussian kernel. Voxel-wise linear regression analysis of the fMRI data employing a canonical hemodynamic response function and serial correlation correction was used to compare the BOLD response during novel to fixation blocks,

restricting the analysis to each hippocampus with the subject-and-session-specific hippocampal masks. The motion correction parameters were included as covariates to account for motion-related signal variance. Voxels surviving this single subject/session analysis at a threshold of $p<0.05$ uncorrected for multiple comparisons were considered active. Activation volumes for the left, right and total hippocampus were calculated by multiplying the number of active voxels by fMRI voxel volume. HPAV was then calculated by dividing the activation volume from the fMRI analysis by total hippocampal mask volume. Thus, left, right and total hippocampal percent activation volume was calculated for each subject in both the saline placebo and insulin conditions.

Whole brain functional analysis (BrainVoyagerQX) was also performed to explore insulin effects outside of the hippocampus. T1-weighted data were re-sampled into 1x1x1 mm isometric voxels in a sagittal orientation and manually warped into a common stereotactic space. (Talairach & Tournoux, 1988) The first few seconds of fMRI data were discarded to allow for image stabilization. Processing of the fMRI data began with sync-interpolated slice timing correction, followed by trilinear 3D motion correction, high-pass temporal filtering (128 sec), smoothing with a 4mm FWHM Gaussian filter, and warping into stereotactic space. (Talairach & Tournoux, 1988) Voxel-wise linear regression analysis of the fMRI data, employing a canonical hemodynamic response function and serial correlation

correction was used to characterize regions engaged in memory encoding by comparing BOLD signal in the novel blocks to fixation. A random effects model was used to characterize whole brain responses in the insulin condition to that of saline in both the control and overall AD groups, as well as across apolipoprotein E-epsilon-4 genotype in the AD subjects. Voxels within the hippocampus and related medial temporal lobe anatomy were considered active if their BOLD time course exhibited a positive or negative correlation with the expected response at $p<0.001$, uncorrected for multiple comparisons. Interactions in BOLD response across apolipoprotein E epsilon-4 genotype were considered significant at $p<0.001$ uncorrected for multiple comparisons as well. Anatomic location and Brodmann classification of activated voxels were characterized with the Talairach Client (www.talairach.org).

Cognitive Testing: A brief test of image recall was performed at the end of the scanning session before subjects were removed from the scanner. A total of 30 images were displayed, (2500 milliseconds each with a 500 millisecond interstimulus gap) 20 of which were previously presented during the fMRI memory stimulus, using the same LCD back projection system. Image recall scores were calculated by subtracting the total number incorrect responses from the total correct. Outside the scanner, a single rater (GPT) administered a battery of standard cognitive test including measures of memory (Wechsler Memory Scale [WMS]–Revised Logical

Memory I and II, (Wechsler, 1955)) Free and Cued Selective Reminding Task, (Grober, 1988)) working memory (Wechsler Adult Intelligence Scale [WAIS] letter–number sequencing (Wechsler, 1955)), executive function (Trailmaking A and B, (34) Verbal Fluency (Hanninen, et al., 1994) [animals, letters R/S and B/L], and visuospatial ability (WAIS Block Design (Wechsler, 1955)). The Mini-Mental State Examination (MMSE) (Folstein, Folstein, & McHugh, 1975) was used as an assessment of general cognition. Individual cognitive performance scores were z-transformed to the overall group mean. The z-transformed scores from similar cognitive domains were then averaged together to create scores for memory, working memory, executive function, visuospatial processing. Individual global cognitive performance was represented by the mean of all z-transformed scores.

Statistical Analysis: Group differences in age, weight, education, fasting glucose, insulin levels and baseline/saline hippocampal percent activation volume were assessed with the Kruskal-Wallis test, while the chi-square test was used to characterize proportions of non-continuous data between groups. The binomial test was used to assess the integrity of blinding with regards to drug administration, as well to assess adequate performance of the task during fMRI scanning. Change in plasma glucose and insulin levels, as well as hippocampal percent activation volume between the placebo and insulin conditions was assessed with the Wilcoxon Signed Ranks test. The Mann-Whitney U test was used to assess APOE-e4-based

differences in changes in hippocampal percent activation volume between the saline and insulin conditions. All statistical analyses outside of imaging space were performed with SPSS 16.0.

Power Analysis

A two-sided, two-sample t-test with $\alpha=0.05$ will have 70% power to detect an effect size ($|\mu_1-\mu_2|/\sigma$) of 1.0 with this sample of 20 subjects with AD and 10 non-demented controls. Similarly, this sample size will have 80% and 90% power to detect effect sizes of 1.12 and 1.30, respectively. This power will likely be reduced if the nonparametric Wilcoxon signed rank test is required for this primary aim instead of the two-sample t-test.

Results

Sample Characteristics (Table 3-1): Ten cognitively intact and 18 early AD subjects participated in this study. Eleven of the early AD subjects were apolipoprotein E-epsilon-4 carriers. The overall control and AD groups were similar in age, education level, and fasting glucose and insulin levels, although there was a trend towards greater body weight in the AD group compared to the controls. Hippocampal percent activation volume in the saline condition was also not statistically different between the control and overall AD group. Demented subjects expectedly exhibited lower global cognitive scores. The sex distribution of the control and AD groups were marginally different, (chi-square=4.68, df=1, p=0.05) with 70% females in the control group, and 28% females in the AD group. Participants were not able to identify the order of drug administration at a rate greater than chance (observed proportion correct in the overall group was 0.18, p=0.001). The apolipoprotein E-epsilon-4 carriers and non-carriers in the AD group were also similar in terms of baseline cognitive performance, age, weight, fasting glucose, educational attainment and HPAV. There were however no female apolipoprotein E-epsilon-4 negative participants. (Table 3-2)

Plasma Glucose: Plasma glucose showed a slight trend towards lower values with insulin in the overall group from 90.7 mg/dl prior to, and 88.7 mg/dl after insulin administration. (p=0.07) None of the participants experienced any hypoglycemia symptoms.

Insulin and Cognitive Performance: The apolipoprotein E-epsilon-4 negative AD group showed improved image recall scores with insulin compared to saline. (mean change = 4, SD 4.16, p=0.05) (Figure 3-2) Neither the apolipoprotein E-epsilon-4 positive AD group nor the control groups demonstrated any change in image recall between the saline and insulin conditions. No insulin-related change in performance on the standardized cognitive tests was noted in the control, overall AD or AD apolipoprotein E-epsilon-4 groups. There was however a negative correlation between performance on the standardized testing and the time delay between insulin administration and the tests, but only in the AD group (Global cognitive score; Pearson correlation -0.734, p=0.001). No similar relationship between drug delivery-testing delay was seen with saline administration.

Hippocampal Percent Activation Volume: Review of logged key responses during the memory encoding task showed that all participants performed at a level greater than chance. (i.e.>50% correct responses) during the fMRI memory stimulus under both the saline (26 out of 28 participants responded correctly >50% of trials, p=0.00) and insulin (26 out of 28 participants responded correctly >50% of trials, p=0.00) conditions. Hippocampal percent activation volume (HPAV) in the saline condition was not statistically different between the control (17.24%, SD 15.85), apolipoprotein E-epsilon-4 negative (9.07% SD 6.12) and apolipoprotein E-epsilon-4 positive AD (16.54% SD 10.66) groups. (chi-square=2.02, df=2, p=0.36)

Insulin-related changes in HPAV are presented in Table 3-2. The control subjects exhibited a trend towards greater HPAV with insulin (21.4%, SD 13.9) compared to saline (17.32, SD 15.9, p=0.074), while HPAV in the overall AD group was not statistically different between the insulin (8.5%, SD 6.2) and saline (13.6%, SD 9.7) conditions (p=0.15). HPAV in the apolipoprotein E-epsilon-4 negative AD subjects was not statistically different between the insulin (12.2%, SD 7.5) and saline (9.1%, SD 6.1) conditions (p=0.31), while apolipoprotein E-epsilon-4 positive AD subjects exhibited decreased HPAV with insulin (6.1&, SD 4.1) compared to saline (16.5%, SD 10.7, p=0.03). (Figure 3-2) Apolipoprotein E-epsilon-4 based differences in changes in HPAV between the saline and insulin conditions in the AD

group were confirmed with the Mann-Whitney U test (Mann-Whitney U=15.00, p=0.03)

Whole Brian fMRI Analysis: Insulin administration did not alter medial temporal lobe activation during the memory task in the control or overall AD group, consistent with the ROI results. In the overall AD group however there was greater BOLD signal in several locations with insulin compared to saline. (Figure 3-3, Table 3-3) These included the anterior cingulate gyrus and prefrontal cortex. Comparing BOLD signal between the saline and insulin conditions across apolipoprotein E-epsilon-4 genotype in the AD group revealed significant differences in functionally related regions that included the left parahippocampal gyrus, left and right precuneus as well as several prefrontal and temporal lobe regions. (Figure 3-4, Table 3-4)

Discussion

The goal for this study was to characterize changes in hippocampal activation in response to insulin in early AD, with the hypothesis that hippocampal activity would be greater during a memory-encoding task with insulin compared to saline. It was further hypothesized that insulin-related changes in hippocampal activity would be greatest in apolipoprotein E-epsilon-4 negative AD subjects. Our ROI-based assessment of hippocampal percent volume activation (HPAV) revealed distinct differences in hippocampal insulin responses based on apolipoprotein E-epsilon-4 genotype in the AD subjects. Carriers of this AD risk factor gene exhibited decreased HPAV with insulin compared to saline during the fMRI memory encoding task, while non-carriers showed no statistically significant change in HPAV with insulin compared to saline. This apolipoprotein E-epsilon-4 genotype based interaction was supported with the whole-brain fMRI analysis, which revealed increased left parahippocampal gyrus activation during the memory stimulus between the saline and insulin conditions in non-carriers compared to carriers.

Image recall performance was improved with insulin in apolipoprotein E-epsilon-4 negative AD subjects in line with earlier reports. These changes correlated with HPAV in this group, linking insulin related changes in cognitive performance and imaging findings according to apolipoprotein E-epsilon-4 genotype in AD. It

was interesting that no insulin-related changes in performance on the standardized cognitive testing was noted, but the negative correlation between scores on these tests and the time delay between insulin administration and testing in AD suggests that insulin might have a short-term effect on memory in AD.

Increased activation was also noted in the overall AD group with insulin compared to saline in several prefrontal regions as well as the anterior cingulate gyrus, areas functionally related to hippocampal function and explicit memory encoding, suggesting that modulation of these regions by insulin might also contribute to the memory facilitation seen in AD in general.

The mechanism underlying insulin' effects on neural memory function are not currently known. Insulin might directly modulate LTP/LTD mechanisms via specific hippocampal insulin receptors as described earlier. Alternatively, insulin signaling might augment oxidative metabolism by promoting the phosphorylation of hexokinase and phosphofructokinase. These enzymes catalyze the initial steps of glycolysis, committing glucose to ATP production. (Hoyer, 2004b) Additionally, other non-specific physiologic effects might also contribute to insulin's effect on memory. Insulin administration has been shown to increase CSF norepinephrine levels that correlate with improvements in cognitive performance. (G. Watson, et al.,

2006) Norepinephrine acts to increase cortical excitability by inhibiting slow acting potassium channels, and might therefore be expected to increase neuronal firing rates, which might increase task attention and performance. Insulin also acts as a vasodilator peripherally, (E. A. Anderson & Mark, 1993) and such an effect on cerebral blood flow, resulting in increased delivery of glucose and oxygen, might be expected to contribute to improved cognitive performance.

How any of these mechanisms might interact with apolipoprotein E-epsilon-4 genotype in their potential effect on neural function or cognitive performance however is not clear. The distinct difference in hippocampal insulin responses across apolipoprotein E-epsilon-4 genotype in AD suggests that these two sub-populations might exhibit fundamental differences in brain insulin sensitivity in AD. It is interesting to note that apolipoprotein E-epsilon-4 negative AD are at increased risk for type 2 diabetes and overweight, (Profenno & Faraone, 2008) providing evidence of peripheral insulin signaling disparities across apolipoprotein E-epsilon-4 genotype. Although the biochemical reasons for this genetic-based difference in insulin brain responses cannot be determined from our study, apolipoprotein E-epsilon-4 genotype in AD nevertheless appears to be an important consideration when assessing insulin effects on both memory and brain activity in AD, and might be an important factor if insulin-based AD treatment is considered in the future.

Several factors must be considered when interpreting our results. The BOLD signal used in fMRI studies is an indirect estimate of underlying neuronal activity. The first link in the chain of events leading to "activation" is the coupling of regional blood flow to neuronal activity. This neurovascular coupling has been shown to exhibit altered temporal characteristics in older adults contributing to reduced signal-to-noise ratio, (D'Esposito, Deouell, & Gazzaley, 2003) and complicating interpretation of functional imaging in this demographic. Furthermore, cholinesterase inhibitors used to treat AD exhibit significant vascular effects independent of neural activity. (D'Esposito, et al., 2003; Rosengarten, Paulsen, Burr, & Kaps, 2008) Most of the AD participants in this pilot study were taking cholinesterase inhibitors, which might have interacted with insulin to alter BOLD signal. Additionally, insulin itself is known to act as a vasodilator peripherally, (E. A. Anderson & Mark, 1993) and thus might alter BOLD-based imaging. Further complications arise from the atrophy seen in AD, which reduces the volume of tissue capable of generating BOLD signal. For this reason, we specifically avoided directly comparing the control and AD groups in imaging space, although the ROI-based hippocampal percent volume activation measurement we used should account for anatomic variation including atrophy.

In summary, our results add to the growing body of research focusing on insulin signaling in early AD by providing evidence that insulin modulates hippocampal function and cognitive performance in early AD according to apolipoprotein E-epsilon-4 genotype. Thus it seems that these two groups should not be combined in studies of insulin in early AD. These observations further suggest that insulin signaling mechanisms might serve as a novel therapeutic target for a subset of AD patients.

Table 3-1-Sample Characteristics

	Normal (n=10)	**Early AD (n=18)**	**p-value**
Global Cognition (Z-score)	0.5 (0.4)	-0.3 (0.8)	<0.001
Percent Female	70%	28%	0.05
Age (years)	73.6 (6.3)	72.6 (7.8)	0.72
Weight (pounds)	149.8 (29.8)	171.9 (28.9)	0.07
Fasting Glucose (mg/dl)	97.9 (12.8)	93.3 (9.3)	0.28
Education (years)	16.4 (2.7)	15.7 (4.2)	0.66
Total HPAV (%) Saline	17.2 (15.6)	13.6 (9.7)	0.53

HPAV; hippocampal activation volume (fMRI active volume at p>0.05/anatomic volume)

Table 3-2-Sample Characteristics by APOE-e4 Genotype in Early AD

	APOE-e4 Negative (n=7)	APOE-e4 Positive AD (n=11)	p-value
Global Cognition (Z-score)	0.5 (0.4)	-0.3 (0.8)	0.82
Percent Female	0%	46%	0.04
Age (years)	73.6 (6.3)	72.6 (7.8)	0.75
Weight (pounds)	149.8 (29.8)	171.9 (28.9)	0.39
Fasting Glucose (mg/dl)	97.9 (12.8)	93.3 (9.3)	0.86
Education (years)	16.4 (2.7)	15.7 (4.2)	0.46
Total HPAV (%) Saline	17.2 (15.6)	13.6 (9.7)	0.11

HPAV; hippocampal activation volume (fMRI active volume at p>0.05/anatomic volume)

Table 3-3. HPAV with saline and insulin by Group. The control group exhibited a trend towards greater HPAV with insulin compared to saline. The APOE-e4 positive AD subjects demonstrated less HPAV with insulin compared to saline.

	Saline	**Insulin**	**p-value**
Control (n=10)	17.24% (15.85	21.43% (13.87)	0.07
Early AD (n=18)	13.64% (9.70)	8.48% (6.24)	0.15
APOE-e4 neg. AD	9.07% (6.12)	12.16% (7.50)	0.31
APOE-e4 pos. AD	16.54% (10.66)	6.13% (4.11)	0.03

Table 3-3-Insulin Effects in Overall AD Group During Memory Encoding Task.

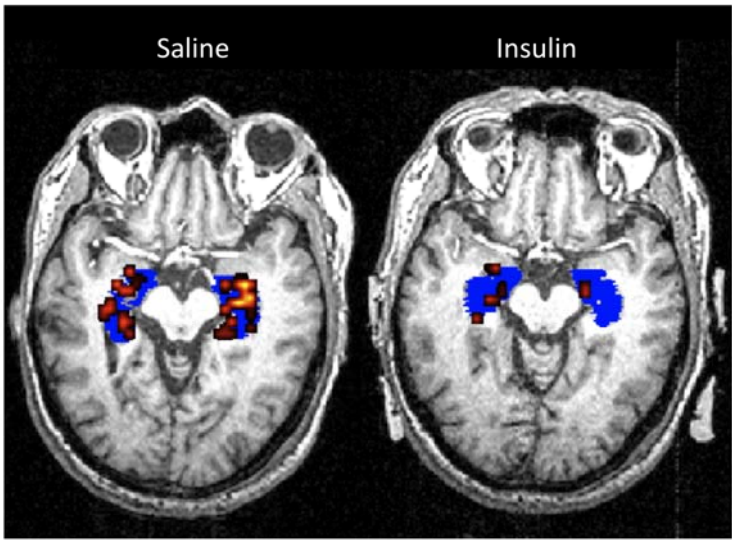
Clusters demonstrating greater BOLD activation with insulin compared to saline in AD subjects.

Lobe	Landmark	X	Y	Z	Brodmann Area	Peak t-value	Cluster Size
Rt. Parietal Lobe	Postcentral Gyrus	55	-26	21	40	4.29	311
Rt. Occipital Lobe	Cuneus	20	-80	4.9	17	5.45	467
Rt. Limbic Lobe	Anterior Cingulate	8.8	33	17	24	5.26	487
Lt. Limbic Lobe	Cingulate Gyrus	0.89	-2.3	29	24	4.62	342
Lt. Occipital Lobe	Lingual Gyrus	-3.8	-81	1.1	18	3.84	309
Lt. Occipital Lobe	Lingual Gyrus	-13	-86	-6.8	18	3.61	357
Lt. Temporal Lobe	Superior Temporal Gyrus	-40	-44	15	13	4.11	301
Lt. Sub-lobar	Insula	-43	-18	19	13	4.32	322

Table 3-4-Apolipoprotein E-epsilon-4 Based Differences in BOLD Activation with Insulin Compared to Saline. Increased activation in the non-carriers compared to carriers between the insulin and saline conditions was observed at the following locations.

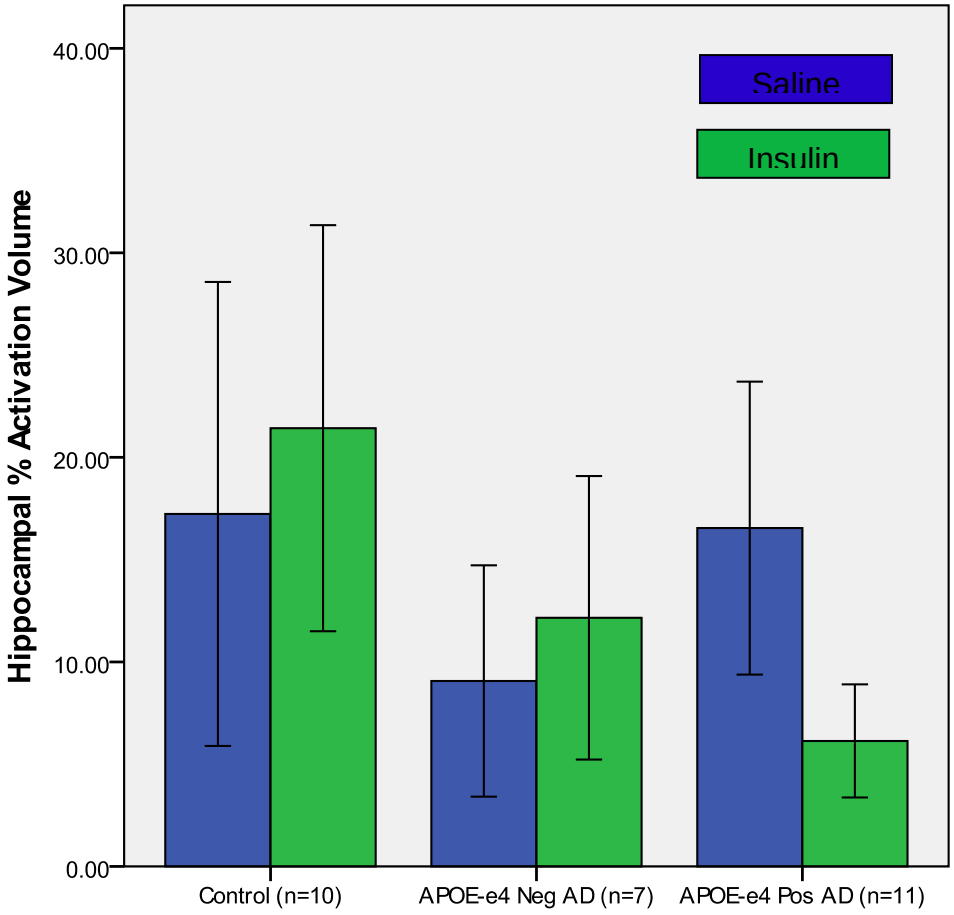
Lobe	Landmark	X	Y	Z	Brodmann Area	Peak t-value	Cluster Size
Rt. Temporal Lobe	Middle Temporal Gyrus	36	-61	28	39	4.65	1738
Rt. Parietal Lobe	Precuneus	12	-61	31	7	6.21	2417
Rt. Occipital Lobe	Lingual Gyrus	18	-67	4	19	5.11	667
Rt. Occipital Lobe	Cuneus	3	-76	19	18	4.68	484
Lt. Temporal Lobe	Superior Temporal Gyrus	-60	-28	4	22	5.30	441
Lt. Temporal Lobe	Superior Temporal Gyrus	-51	-55	16	22	4.45	433
Lt. Sub-lobar	Thalamus	-24	-25	10		3.89	392
Lt. Parietal Lobe	Inferior Parietal Lobule	-36	-52	46	40	4.49	587
Lt. Parietal Lobe	Precuneus	-6	-61	46	7	4.86	576
Lt. Occipital Lobe	Precuneus	-21	-67	22	31	3.97	440
Lt. Limbic Lobe	Posterior Cingulate	-6	-58	10	30	5.30	678
Lt. Limbic Lobe	Posterior Cingulate	-6	-37	16	29	4.31	665
Lt. Limbic Lobe	Parahippocampal Gyrus	-30	-25	-17	35	4.75	351
Lt. Frontal Lobe	Precentral Gyrus	-54	-13	37	4	4.03	501

Figure 3-1-Hippocampal Percent Activation Volume



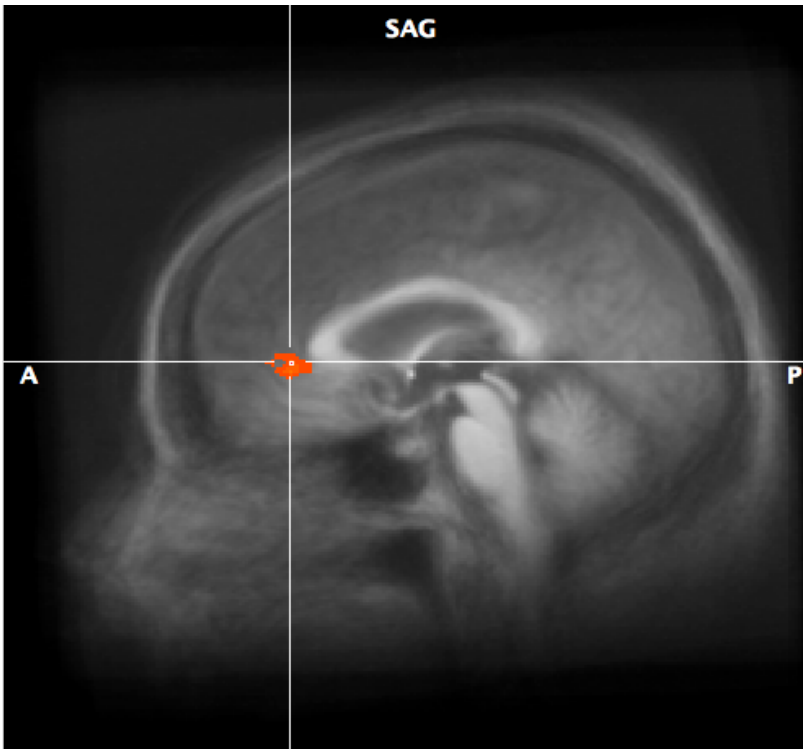
Analysis of BOLD data was restricted to the hippocampus by session-specific masks (blue). The volume of active voxels (red) was expressed as a percent of mask volume.

Figure 3-2-Insulin Modulation of Hippocampal Activation.



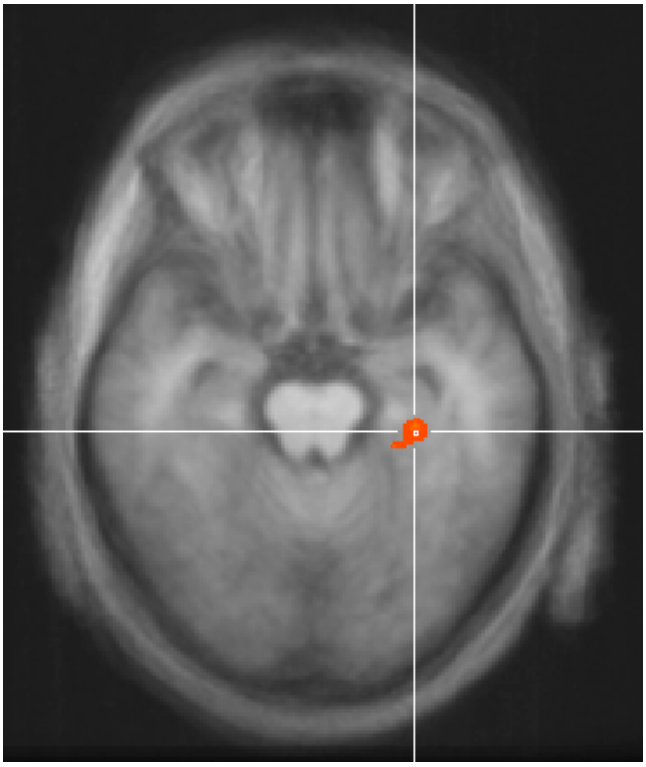
There was a trend towards more HPAV in controls with saline compared to insulin. Total HPAV decreased between the saline and insulin conditions in the APOE-e4 positive AD group, while non-carriers showed a non-significant increase. Error bars represent 95% confidence intervals

Figure 3-3-Insulin Effects in the Overall AD Group.



BOLD activation during the memory encoding task was increased with insulin compared to saline in the overall AD group at several locations outside the medial temporal lobes, including the anterior aspect of the cingulate gyrus. Random effects analysis, image thresholded at $p<0.02$, $k>13$ for illustrative purposes

Figure 3-4-APOE-e4-based Differences in Insulin Responses at the Left Parahippocampal Gyrus.



There was an interaction across APOE-e4 genotype in the AD group with regard to responses to insulin at the left parahippocampal gyrus. Random effects analysis, image thresholded at $p<0.02$, $k>13$ for illustrative purposes.

Chapter 4-Insulin and Motor Activity in Early Alzheimer's Disease

Insulin and Motor Activity in Early Alzheimer's Disease

Abstract

Insulin administration appears to facilitate memory in early Alzheimer's disease, but neither the anatomic correlates nor the mechanisms underlying this phenomenon are known. Insulin might act specifically at hippocampal insulin receptors to modulate memory performance, but insulin might also act to improve memory via less specific means. The goal of this experiment was to characterize insulin responses during a non-memory task with functional MRI. It was hypothesized that motor activation would be increased with insulin compared to saline in all subjects. The results of this experiment demonstrate that insulin might modulate non-memory related function in AD subjects, and that motor responses to insulin might differ according to apolipoprotein E-epsilon-4 genotype. The results of this experiment therefore suggest that extra-hippocampal effects of insulin might contribute to the memory facilitation seen in Alzheimer's disease with insulin.

Background

Several reports of acute improvements in cognitive performance in AD after experimental insulin administration suggest that insulin might modulate memory processes, (S. Craft, et al., 2000; S. Craft, et al., 1999; Craft, et al., 1993; Craft, et al., 1996; M. Reger, et al., 2006; G. S. Watson & Craft, 2004) Insulin's effect on cognitive performance also appears to be modulated by apolipoprotein E-epsilon-4 genotype, with improved recall in non-carriers after a single 40IU dose delivered via intranasal spray, while carriers demonstrated impaired recall. (M. Reger, et al., 2006)

The neuroanatomic correlates mediating this phenomenon are not established, although the hippocampus is a likely candidate. Insulin receptors are found throughout the brain, but are most concentrated in select regions including the hippocampus and entorhinal cortex. (Hoyer, 1993; Liang & Chaung, 2006; Messier & Teutenberg, 2005b; M. Reger, et al., 2006) Insulin has been shown in cell cultures to modulate the activity of NMDA receptors (Christie, et al., 1999) as well as the surface expression of AMPA receptors. (Ahmadian, et al., 2004) It seems possible therefore that insulin might act specifically at the hippocampus to modulate LTP and LTD via alterations of NMDA and AMPA currents, translating to changes in synaptic activity and ultimately cognitive performance.

How apolipoprotein E-epsilon-4 genotype might interact with insulin in terms of cognitive enhancement is not clear. Glucose utilization has been shown to differ across apolipoprotein E-epsilon-4 genotype in AD, with less insulin-mediated glucose disposal in AD non-carriers compared to both AD carriers and control subjects during experimental hyperinsulinemia. (S. Craft, et al., 1999) It seems therefore that the influence of the apolipoprotein E-epsilon-4 allele might modulate neuronal physiology during non-memory tasks in addition to the previously described effects on memory.

The experiments described in Chapter 3 demonstrate that insulin modulates hippocampal function and cognitive performance in early AD according to apolipoprotein E-epsilon-4 genotype. Insulin however might influence neuronal function outside of the hippocampus and still contribute to changes in cognitive performance. The goal of this study was to determine if insulin administration was associated with changes in brain activation during a simple fMRI motor task in early AD. We hypothesized that fMRI activation in motor cortex would increase with insulin compared to saline in both control and AD subjects. Based on the lack of data to suggest apolipoprotein E-epsilon-4 genotype modulates non-memory effects of insulin, we hypothesized that motor responses between the saline and insulin conditions would be similar across apolipoprotein E-epsilon-4 genotype.

Methods

Subjects: All participants were right-handed non-diabetics over the age of 60, and provided written informed consent in accordance with the University of Kansas Medical Center Human Subjects Committee. Diagnostic criteria for AD included gradual and progressive memory impairment and deficit in at least one other cognitive domain. (L Berg, et al., 1988) A thorough exam, including an interview with a collateral source familiar with the subject, was performed to characterize the presence and severity of dementia with the Clinical Dementia Rating (CDR) scale, (Morris, 1993) focusing on intrasubject change, rather than deviation from group norms. (Morris, 1993, 1997; Rockwood, et al., 2000) Participants included AD subjects with CDR scores of 0.5 and 1, while those with CDR scores of 0 served as controls. Exclusion criteria (Burns, et al., 2007) included diabetes (defined as self-reported insulin or oral hypoglycemic agent use, or fasting blood glucose > 126 mg/dl), disorders other than AD with the potential to impair cognition, stroke, clinically significant depressive symptoms, abnormalities in vitamin B12 levels, positive rapid plasma reagin, abnormal thyroid function, or concurrent use of psychoactive or investigational medications. All participants underwent fMRI scanning twice, once after insulin administration, and once after saline placebo in a double-blind, counterbalanced crossover fashion.

Insulin Administration: A single forty IU dose of insulin aspart (Benedict, et al., 2007) or an equivalent volume of saline placebo was administered via nasal spray (M. A. Reger & S. Craft, 2006; M. A. Reger, et al., 2006) approximately 30 minutes prior to the fMRI motor task. Blood samples were obtained from the left antecubital vein prior to insulin administration just before and again about 1 hour after insulin or placebo administration to characterize changes in peripheral glucose in response to insulin administration.

Functional MRI Motor Task: A simple fMRI motor task was employed in this study. Each of the 5 active blocks consisted of a green circle on a black background presented 5 times for a duration of 2 seconds, with a 2 second interstimulus gap. A red circle replaced the green circle for the rest blocks, which were otherwise identical to the active blocks in timing and duration. Text clues ("Move" and "Rest") were included for each stimulus to serve as a reminder of the task. Subjects were instructed to firmly squeeze a soft rubber bulb with their right hand when the green circle was presented, and to rest when the red circle was displayed. The entire motor stimulus was 3 minutes 28 seconds in duration. Stimuli were presented with commercial software (Presentation, NeuroBehavioral Systems) using an LCD back projection system with MRI-compatible vision correction when necessary.

Functional MRI: All imaging was performed with a Siemens 3T Allegra scanner using a quadrature head coil. A T1-weighed (Magnetization-Prepared Rapid Gradient Echo [MPRAGE]; TR=2500 ms, TE=4.38 ms, TI=1100ms, flip angle=8 degrees, field of view 256x256mm with 18% oversample, slice thickness/gap/number=1mm/0/208) anatomic image was acquired to provide a backdrop on which to overlay functional data. Blood-Oxygen Level Dependent (BOLD; FOV=240x100, slice thickness/gap/number=5.0mm/0mm/25, TR=2000, TE=50, FA=90, 102 data points) sensitive EPI data were acquired parallel to the AC-PC line during performance of the motor task.

Functional MRI Data Analysis: BOLD MRI data were assessed with BrainVoyagerQX. T1-weighted data were re-sampled into 1x1x1 mm isometric voxels in a sagittal orientation and warped into a common stereotactic space. (Talairach & Tournoux, 1988) The first 8 seconds of fMRI data were discarded to allow for T1 stabilization. Processing of the fMRI data began with sync-interpolated slice timing correction, followed by trilinear 3D motion correction, high-pass temporal filtering, (128 sec.) smoothing with a 4mm FWHM Gaussian filter, and warping into sterotactic space. (Talairach & Tournoux, 1988) Voxel-wise linear regression analysis of the fMRI data, employing a canonical hemodynamic response function and serial correlation correction, was used to compare the BOLD response during motor activity to that of the rest blocks. A random effects model was used to

characterize whole brain responses in the insulin condition to that of saline in both the control and overall AD groups, as well as across apolipoprotein E-epsilon-4 genotype in the AD subjects. Voxels within the primary motor cortex, ipsilateral motor cortex or supplemental motor area were considered active if their BOLD time course exhibited a positive or negative correlation with the expected response at $p<0.001$, uncorrected for multiple comparisons. Interactions in BOLD response across apolipoprotein E epsilon-4 genotype were considered significant at $p<0.001$ uncorrected for multiple comparisons as well. Anatomic location and Brodmann classification of significant voxels were characterized with the Talairach Client (www.talairach.org).

Statistical Analysis: Group differences in age, weight, education, fasting glucose and insulin levels were assessed with the Kruskall-Wallis test, while the chi-square test was used to characterize proportions of non-continuous data between groups. Differences in plasma glucose and insulin levels between the placebo and insulin conditions were assessed with Wilcoxon Signed-Ranks test. All statistical analysis outside of imaging space was performed with SPSS 16.0.

Results

Sample Characteristics: Ten cognitively intact control subjects and 18 early AD subjects were enrolled and completed the study, but one of the AD subjects exhibited excessive motion during both fMRI sessions and this data was excluded from analysis. Ten of the remaining AD subjects carried at least 1 apolipoprotein E-epsilon-4 allele, while 7 AD subjects were apolipoprotein E-epsilon-4 negative. Baseline peripheral glucose and insulin levels were similar in all groups. Sample characteristics are presented in Table 4-1. The sex distribution between the control (70% female) and AD (24%) groups was unequal. (chi-square 5.6, df=1, p=0.02) The drug order randomization was equal between the two groups. (chi-square 1.3, df=1, p=0.23)

Plasma Glucose: Plasma glucose was marginally statistically lower (p=0.05) after insulin administration, lowering by about 2 mg/dl from a value of 91.3 mg/dl before insulin to 89.1 mg/dl after. None of the participants however experienced any hypoglycemia symptoms.

Functional MRI Results: The motor task produced the expected BOLD activation in the contralateral (left) primary motor cortex, as well as at the ipsilateral (right) motor cortex and supplemental motor area in both the control and AD groups. Random effects analysis comparing motor responses between the saline and insulin conditions revealed no differences in the control group. Evaluation of imaging data in the overall AD group showed several regions which exhibited greater BOLD activation with insulin compared to saline (Table 4-2), but none were found within the primary motor cortex, ipsilateral motor cortex or supplemental motor area. Comparison of insulin-related changes in BOLD motor responses across apolipoprotein E-epsilon 4 genotype in AD did reveal increased activation at the ipsilateral precentral gyrus (x, y, z = 36, -19, 40) in the non-carriers compared to the carriers. (Figure 4-1)

Discussion

The results of this preliminary study suggest apolipoprotein E-epsilon-4 based differences in BOLD responses to insulin during a simple motor task in AD. Apolipoprotein E-epsilon-4 negative AD persons are at increased type 2 diabetes risk compared to carriers, suggesting differences in peripheral insulin sensitivity across apolipoprotein E-epsilon-4 genotype, but how this might translate to the central nervous system is not clear. The differences occurred at the ipsilateral motor cortex, which is though to contribute about ten percent of the total cortical motor output during such tasks, although why no differences were observed at the site of greatest activity, the contralateral motor cortex, is not clear.

Several explanations might offer insight in our observations. As mentioned earlier, insulin increases CSF norepinephrine levels, (G. Watson, et al., 2006) possibly by modulating NE transporter mRNA transcription at the locus ceruleus (Figlewicz, Szot, Israel, Payne, & Dorsa, 1993) via a MAP kinase pathway. (Kusari, Byon, Bandyopadhyay, Kenner, & Kusari, 1997) Noradrenergic signaling increases cortical excitability by inhibiting slow-acting potassium channels, resulting in decreased after-hyperpolarization. (Andersen, Morris, Amaral, Bliss, & O'Keefe, 2007) Therefore, insulin administration might increase task-related BOLD signal by modulating cortical excitability via increasing CSF norepinephrine levels.

Insulin's potential effects on cortical excitability however might be more complex. Motor cortex hyperexcitability is a feature of Alzheimer's disease, and might be mediated by a shift in balance of glutamate signaling from NMDA to non-NMDA receptor types, such as the kainate receptor. (Di Larzzaro, et al., 2003; Di Larzzaro, et al., 2004) Insulin and IGF-1 have been shown to prevent kainite neurotoxicity via a PI3-K dependent pathway. (Leski, Valentine, Baer, & Coyle, 2000) One of insulin's actions might therefore include attenuation of cortical excitability by modulation of kainite signaling. However, reduced IRS-1-related PI3-K levels have been described in AD (Steen, et al., 2005), suggesting that insulin's inhibitory effect on cortical excitability via kainite receptors might be attenuated in AD. The balance therefore between insulin's facilatory and inhibitory mechanisms regulating cortical excitability might be upset in AD, leading to increased excitability in response to insulin.

Alternatively, insulin's effect on motor activation might reflect insulin-induced facilitation of oxidative metabolism. Insulin promotes trapping of glucose within neurons by phosphorylation of hexokinase and phosphofructokinase, initiating oxidative metabolism and leading ultimately to ATP production. Attenuated glucose metabolism has been reported in AD, (Fukuyama, et al., 1994) and insulin-related increases in oxidative metabolism and ATP production might therefore translate into increased synaptic activity and task-related BOLD signal in AD subjects.

How these mechanisms might interact with the apolipoprotein E-epsilon-4 allele is however not clear. It is noteworthy however that the apolipoprotein E-epsilon-4 genotype appears to interact with β2 polymorphisms to confer increased AD risk. These polymorphisms also exhibit different signaling qualities, (Yu, et al., 2008) suggesting that noradrenergic mechanisms might differ across apolipoprotein E-epsilon-4 genotype. It has also been that the antidepressant mirtazapine, which inhibits the reuptake of both norepinephrine and serotonin, appears to work faster in apolipoprotein E-epsilon-4 positive subjects compared to non-carriers. Thus the apolipoprotein E-espilon-4 based differences described might suggest that insulin's effects on neuronal physiology are mediated by noradrenergic mechanisms.

Insulin is also known to act as a vasodilator, (E. A. Anderson & Mark, 1993) but increased regional blood flow would be expected to decrease rather than increase BOLD signal as we describe. Hyperinsulinemia has been shown to be associated with decrease BOLD signal during visual stimulation, (Seaquist, et al., 2007) attributed to increased regional blood flow. It seems unlikely therefore that the increased BOLD signal we observed is driven by insulin's vasodilatory properties, but it is possible that such effects might contribute in some fashion to our findings.

In summary, compared to saline, a single 40IU dose of insulin appears to modulate ipsilateral motor cortex BOLD signal during a simple motor task according to apolipoprotein E-epsilon-4 genotype in AD. In the context of understanding insulin's effect on cognitive performance in AD, this suggests that insulin might act to improve memory by modulating widespread cortical functions, rather than by acting specifically at temporal lobe structures, such as the hippocampus, to produce cognitive enhancement. Reports of apolipoprotein E-epsilon-4 based differences in noradrenergic signaling suggest that insulin might act via noradrenergic mechanisms. Observations of increased CSF norepinephrine levels would support this idea as well.

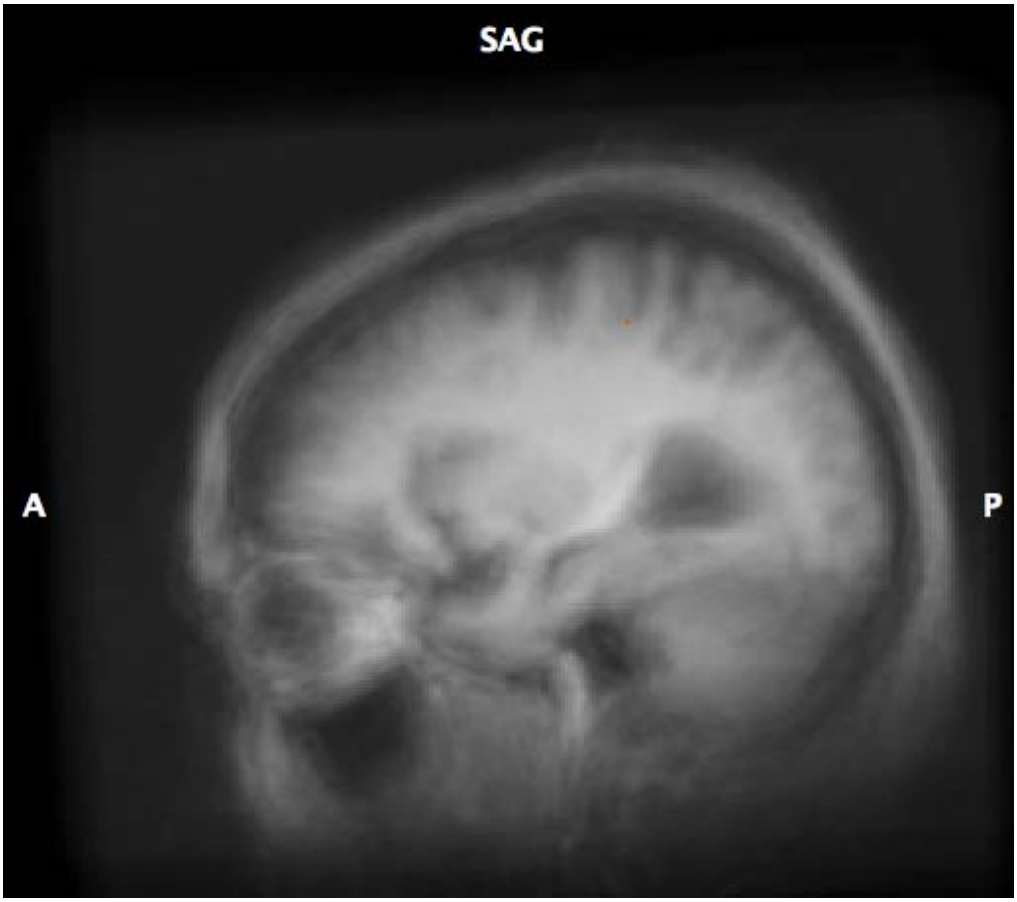
Table 4-1. Sample Characteristics

	Control (n=10)	**Early AD (n=18)**	**p-value**
Age (years)	73.6 (6.3)	72.5 (8.0)	0.65
Fasting Plasma Glucose (mg/dl)	97.9 (12.8)	94.0 (9.1)	0.28
Weight (lbs)	149.8 (29.8)	173.8 (28.7)	0.05
MMSE	29.3 (1.3)	24.8 (4.6)	<0.001

Table 4-2-Clusters Demonstrating Greater Motor Activation With Insulin Compared to Saline in AD.

Lobe	Landmark	X	Y	Z	Brodmann Area	Peak t-value	Cluster Size
Rt. Parietal Lobe	Postcentral Gyrus	55	-26	21	40	4.29	311
Rt. Occipital Lobe	Cuneus	20	-80	4.9	17	5.45	467
Lt. Limbic Lobe	Anterior Cingulate	8.8	33	17	24	5.26	487
Lt. Limbic Lobe	Cingulate Gyrus	0.89	-2.3	29	24	4.62	342
Lt. Occipital Lobe	Lingual Gyrus	-3.8	-81	1.1	18	3.84	309
Lt. Occipital Lobe	Lingual Gyrus	-13	-86	-6.8	18	3.61	357
Lt. Temporal Lobe	Superior Temporal Gyrus	-40	-44	15	13	4.11	301
Lt. Sub-lobar	Insula	-43	-18	19	13	4.32	322

Figure 4-1-Insulin-Related Differences in Motor Activation Across APOE-e4 Genotype in Early AD



Apolipoprotein E-epsilon-4 negative AD subjects exhibited greater insulin-related activation change compared to non-carriers at the right pre-central gyrus during a simple motor task. (Random effects analysis, $p<0.001$ uncorrected for multiple comparisons)

Chapter 5-Intranasal Insulin Does Not Alter Mean Cerebral Blood Flow

Intranasal Insulin Does Not Alter Mean Cerebral Blood Flow

Abstract

Insulin administration appears to facilitate memory in early Alzheimer's disease, but the mechanisms underlying this phenomenon are not known. Insulin is known to act as a vasodilator peripherally, and such an effect if it occurs centrally might contribute to insulin-related cognitive enhancement. Insulin-related changes in mean cerebral blood flow might also confound blood oxygen level based functional MRI experiments investigating insulin's effect on cognition. Mean cerebral blood flow was assessed with arterial spin-labeled MRI after intranasal saline and insulin administration, hypothesizing that mean cerebral blood flow would increase with insulin in all subjects. Our experiment found no insulin-related changes in mean cerebral blood flow after a single 40IU dose of insulin delivered via nasal, suggesting that insulin's effects on cognitive performance are not mediated by global changes in cerebral perfusion.

Background

Several reports of acute improvements in cognitive performance in AD after experimental insulin administration suggest that insulin might modulate memory processes, although the mechanisms related to this phenomenon are not clear. While insulin receptors are found throughout the brain, most are concentrated in select regions including the hippocampus and entorhinal cortex (Hoyer, 1993; Liang & Chaung, 2006; Messier & Teutenberg, 2005a; Seaquist, et al., 2007) where insulin might modulate long term potentiation (LTP) and long-term depression (LTD) mechanisms. ((Ahmadian, et al., 2004; Christie, et al., 1999) It therefore seems possible that insulin might directly modulate hippocampal function to facilitate memory.

Insulin however also possesses vasodilatory properties peripherally, acting at endothelium to increase nitric oxide (NO) production. (E. A. Anderson & Mark, 1993) Such vasodilatory effects could contribute to insulin effect on memory by increasing delivery of glucose and oxygen to metabolically active areas either the regionally or globally, if they in fact pertain to cerebral blood flow.

Reductions in parietal-temporal and frontal blood flow in AD have been reported, and these appear to be related to clinical presentation in early AD. (Postiglione, Lassen, & Holman, 1993) Right-sided flow deficits for example are associated with visuospatial apraxia, suggesting that alterations in cerebral blood flow may be an important aspect of disease expression. Cilostazol, a PDE3 phosphodiesterase inhibitor that acts as an antiplatelet agent as well as a vasodilator, used in conjunction with cholinesterase inhibitors has been shown to lead to improved MMSE scores in AD after 5-6 months, (Arai & Takahashi, 2009) suggesting that increasing cerebral blood flow might improve cognitive performance in AD. It is not known how or if cerebral flow deficits in AD respond to insulin, but a possible mechanism underlying reports of insulin-related cognitive enhancement might involve changes in cerebral blood flow.

Insulin's vasodilator action also has the potential to confound BOLD-based functional MRI (fMRI) studies. The BOLD effect is a complex phenomenon based in the physiologic response of blood vessels to adjacent brain activity. In the simplest case, the identification of brain "activity" with BOLD fMRI depends in large part on the difference in blood flow between two states, such as "rest" and "active" in a region over time. Larger differences in blood flow translate to greater BOLD activation. The degree of BOLD signal change between the "rest" and

"active" states in a typical experiment is small, on the order of 1-2%. Vasodilators such as acetoazolamide tend to cause a persistent increase in basal blood flow (Vagal, Leach, Fernandez-Ulloa, & Zuccarello, 2009) and minimize the differences in regional blood flow between contrasting conditions (i.e. "rest" and "active) in response to task-related modulation of neuronal activity. Experimentally-induced hyperinsulinemia has been shown to decrease BOLD signal during visual stimulation, with no change in neuronal activity as measured by visually evoked potentials. (Seaquist, et al., 2007) This disparity between imaging modalities was thought to be due to insulin-related vasodilation. Characterizing any insulin-related changes in cerebral blood flow therefore might be important when interpreting BOLD imaging of insulin effects.

The method of delivery however might influence the vasodilatory effects of insulin. Insulin administered via IV has ready access to endothelium, where it can promote NO production, but how insulin given via nasal spray might behave in this regard is not known. Small peptides delivered via nasal spray appear to bypass the peripheral circulation and directly access the CSF by traveling along trigeminal nerves. Insulin administered in this manner would presumably have to traverse nearly the entire thickness of the vascular wall to reach endothelium and promote

vasodilation. It seems therefore that insulin delivered via nasal spray might not be as effective in altering blood flow as insulin delivered via IV might be.

Because insulin-induced changes in blood flow might contribute to cognitive performance and confound BOLD imaging, mean cerebral blood flow after both saline and insulin administration was measured with arterial spin-labeled MRI (ASL-MRI) in 8 cognitively intact older adults and 13 early AD subjects. It was hypothesized that mean cerebral blood flow would be greater after insulin administration compared to that in the saline condition. Based on earlier PET reports, it was anticipated that the AD group would demonstrate reduced cerebral blood flow relative to controls in the saline condition. (Postiglione, et al., 1993)

Methods

Subject Selection: All participants were right-handed non-diabetics over the age of 60, and provided written informed consent in accordance with the University of Kansas Medical Center Human Subjects Committee. Diagnostic criteria for AD included gradual and progressive memory impairment and decline in at least one other cognitive domain. (L Berg, et al., 1988) A thorough exam, including an interview with a collateral source familiar with the subject, was performed to characterize the presence and severity of dementia with the Clinical Dementia Rating (CDR) scale, (Morris, 1993) focusing on intrasubject change rather than deviation from group norms. (Morris, 1993, 1997; Rockwood, et al., 2000) Participants included AD subjects with CDR scores of 0.5 and 1, while those with CDR scores of 0 served as controls. Exclusion criteria (Burns, et al., 2007) included diabetes (defined as self-reported insulin or oral hypoglycemic agent use, or fasting blood glucose > 126 mg/dl), disorders other than AD with the potential to impair cognition, stroke, clinically significant depressive symptoms, abnormalities in vitamin B12 levels, positive rapid plasma reagin, abnormal thyroid function, or concurrent use of psychoactive or investigational medications. Apolipoprotein E genotype in the AD subjects was determined by allelic discrimination. Participants were not excluded from this pilot if they were currently taking AD medications such as cholinesterase inhibitors or NMDA agonists.

All participants underwent MRI scanning on two occasions at least 48 hours apart in a double-blind, crossover, counterbalanced manner. All scanning was performed in the morning after an overnight fast. Participants were instructed to take any morning medications as usual, and to maintain their usual caffeine intake on study days. For each visit, either 40IU insulin aspart, (Benedict, et al., 2007) or an equal volume of saline placebo, was administered via nasal spray. (M. A. Reger & S. Craft, 2006) An IV line was placed prior to insulin administration for pre and post-drug/placebo glucose and insulin testing, and for ready vascular access for dextrose infusion if necessary due to hypoglycemia.

Image Acquisition: A Siemens 3T Allegra scanner using a quadrature head coil was used for all imaging. Following routine localizer scans for anatomic orientation, 2D time-of-flight MR angiography was used to visualize carotid artery anatomy within the upper cervical region. The distance between the isocenter of the magnet and the junction of the cavernous and cerebral portions of the internal carotid arteries was recorded and used to adjust the label offset distance parameter for each subject. This step was to minimize differences in labeling efficiency related to variability in head placement within the bore of the scanner.

Cerebral Blood Flow; Arterial Spin Labeling (ASL-MRI): Forty labeled and 40 unlabeled image pairs were acquired perpendicular to the magnet axis over a period of 5 minutes, 34 seconds. (FOV=240mmx100mm, slice thickness/gap/number=8.0mm/2.0mm/10, TR=4000, TE=16.0, FA=90) The DICOM data were converted to ANALYZE format, and the 40 unlabeled images were averaged into a single mean unlabeled image file using the *imagecalc* function of SPM5. Similarly, the 40 labeled images are averaged into a single mean labeled image.

The mean signal intensity from four identical 10mm spherical regions, (left frontal, right frontal, left occipital, right occipital) including both gray and white matter (Figure 5-1), in each of the mean labeled and mean un-labeled image was recorded and used to calculate regional blood flow at each of the 4 locations according to previously published reports. (Wang, et al., 2003) The 4 regional cerebral blood flow values were averaged to provide an estimate of mean cerebral blood flow for the session.

Statistical Analysis: Group differences in age, weight, education, fasting glucose and baseline mean cerebral blood flow were assessed with Kruskall-Wallis test, while the chi-square test was used to characterize proportions of non-continuous

data between groups. Differences in plasma glucose and mean cerebral blood flow between the placebo and insulin conditions were assessed with the Wilcoxon Signed Ranks test. All statistical analysis was performed with SPSS 16.0.

Results

The control and AD groups were similar in terms of age, fasting glucose and baseline mean cerebral blood flow, although there was a trend towards greater weight in the AD group. (Table 5-1) The groups were different however in sex distribution, with 25% females in the control group, and 77% females in the AD group. (chi-square 5.45, df=1. p=0.03) The order of drug administration was similar between the groups. (chi-square 1.15, df=1, p=0.27)

Mean plasma glucose in the overall group was similar before (90.7 mg/dl, SD 13.1) and after (88.9 mg/dl, SD 12.6) insulin administration (p=0.09) suggesting there may have been a slight and physiologically negligible trend towards lower plasma glucose levels with insulin.

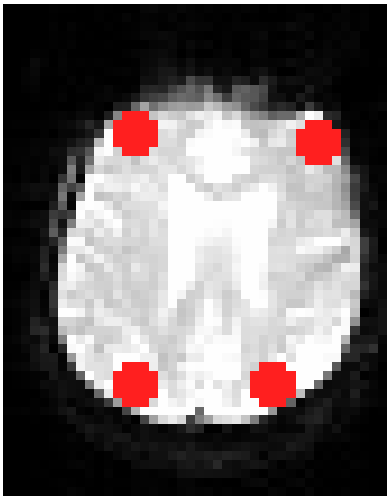
The values obtained for mean cerebral blood flow were higher than the expected value of 50 ml/100g/min. Mean CBF in the overall group during the saline condition for example was 81.83 ml/100g/min (one-sample t-test for saline mean cerebral blood flow vs. 50 ml/100g/min, $p<0.001$) Mean cerebral blood flow in the overall group was the same in both the saline (81.83 ml/100g/min, SD 26.8 mg/100g/min) and insulin (82.89 mg/100g/min, SD 25.9 mg/100g/min) conditions. (p=0.90) Mean cerebral blood flow in the control group was not significantly

different between the saline (77.0 ml/100g/min, SD 16.4 mg/100g/min) and insulin (84.1 ml/100g/min, SD 30.8 mg/100g/min) conditions. (p=0.56) Mean cerebral blood flow in the AD group was also not significantly different in the saline (84.8 ml/100g/min, SD 31.9 mg/100g/min) and insulin (82.1 ml/100g/min, SD 23.8 mg/100g/min) conditions. (p=0.75) Furthermore, there were no significant apolipoprotein-E-epsilon-4 based differences in insulin-related change in mean CBF (Chi-square 0.09, df=1, p=0.77)

Table 5-1. Sample Characteristics

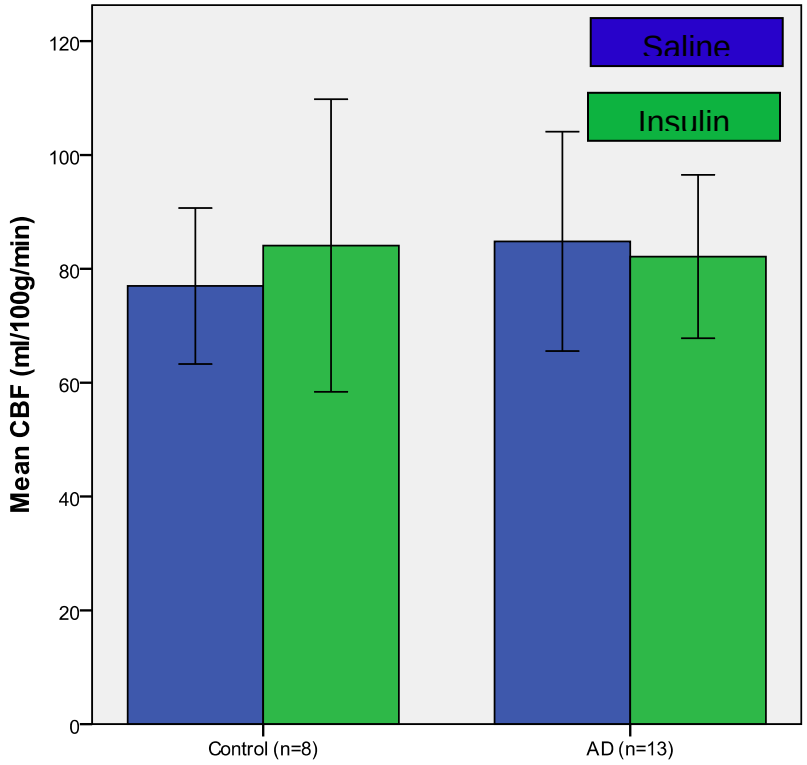
	Control (n=8)	Early AD (n=13)	p-value
Age (years)	73.3 (5.7)	71.2 (8.2)	0.70
Weight (lbs.)	152.9 (32.7)	171.8 (27.1)	0.07
Fasting Glucose (mg/dl)	99.2 (14.2)	91.1 (8.8)	0.22
Mean CBF (ml/110g/min, saline condition)	77.0 (16.4)	84.8 (31.9)	0.47

Figure 5-1-Measurement of Cerebral Blood Flow.



Signal intensity from 4 regions (red) was measured for both mean labeled and un-labeled ASL-MRI images to calculate mean cerebral blood flow.

Figure 5-2-Mean Cerebral Blood in Control and Early AD Subjects.



No change in mean cerebral blood flow was evident between the saline and insulin conditions in either the overall group or the separate diagnosis groups (shown). Error bars represent 95% confidence intervals.

Discussion

Our results show that a single 40IU dose of insulin aspart does not appear to alter mean cerebral blood flow as measured with ASL-MRI. Our results however must be interpreted with several caveats in mind. First, the cerebral blood flow measurements were delayed about 30 minutes after drug administration while subjects were positioned within the scanner and the initial localizer scans obtained. The CSF pharmacokinetics of insulin administered via nasal spray are not well understood, and it is possible that cerebral blood flow measurements made earlier (or later) might have yielded different results. Second, the mean cerebral blood flow values are greater than the generally agreed upon mean cerebral blood flow value of 50ml/100g/min, and furthermore do not appear to be in agreement with other studies that demonstrate reduced cerebral blood flow in AD. This would suggest that the mean cerebral blood flow measurement as performed might not be accurate or sensitive enough to produce meaningful measures of blood flow. Our ASL measurement appears to exhibit quite a bit of variability as well. The 95% confidence interval of the mean difference in mean cerebral blood flow between the saline and insulin conditions is -9.47-11.59 ml/100g/min, suggesting that ASL-MRI as performed might not be an adequately sensitive measure for small samples such as this. However, we measured resting mean cerebral blood flow, rather than task-related changes in regional cerebral blood flow. It might be that insulin modulates task-related changes in regional blood flow. ASL-MRI may be better suited to

detecting task-related changes in regional blood flow, rather than mean cerebral blood flow during rest.

The lab results suggest that there might have been a decrease in plasma glucose in the insulin condition relative to saline, however plasma glucose was still well above the lower limits of normal, and none of the subjects experienced symptoms of hypoglycemia. A larger sample might clarify this discrepancy with earlier reports that intranasal insulin does not influence peripheral glucose.

In summary, mean cerebral blood flow in our sample did not appear to change with insulin, however it is not clear if this indicates that intranasal insulin does not influence mean cerebral blood flow, or that the ASL-MRI method we used is not sensitive enough to detect change.

Table 5-1. Sample Characteristics

	Control (n=8)	AD (n=13)	p-value
Age (years)	73.3 (5.7)	71.2 (8.2)	0.54
Percent Female	25	77	0.03
Weight (lbs.)	152.9 (32.7)	171.8 (27.1)	0.19
Fasting Glucose (mg/dl)	99.2 (14.2)	92.1 (8.8)	0.18
Baseline mean CBF (ml/100g/min)	77.0 (16.4)	84.8 (31.9)	0.47
MMSE	29.3 (1.3)	24.8 (4.6)	<0.001

Chapter 6-Summary of Findings and Discussion

Summary of Findings

Chapter 2-Hippocampal and Limbic Volume Are Related to Insulin Levels in Early Alzheimer's Disease

Peripheral insulin appears to positively correlate with whole brain volume in early AD. (Burns, et al., 2007) The goal of this experiment was to examine this relationship on a regional basis. It has been postulated that insulin signaling might act to modulate pathologic burden in AD by inhibiting the formation of both Abeta-42 and neurofibrillary tangles. Brain volume loss as assessed with MRI frequently used to quantify pathologic burden in AD. (Borthakur, et al., 2008; Callen, et al., 2001; Clark, et al., 2008; Hall, et al., 2008) Therefore, if insulin acts to attenuate AD pathologic burden, insulin levels should positively correlate with volume in regions known to possess insulin receptors, in particular the hippocampus. We found a positive correlation between peripheral insulin level and hippocampal volume, as well as that of paralimbic structures, in the overall AD group without regard for apolipoprotein E-epsilon-4 genotype. This might represent either a neurotrophic or neuroprotective influence of insulin on insulin-sensitive anatomy, and provides additional evidence that insulin may be an important factor in brain health in AD. Thus, insulin might confer benefit in terms of preservation of hippocampal volume in

AD, although longitudinal studies that monitor changes in hippocampal volume over time a necessary to define insulin's influence on brain structure.

Chapter 3-Insulin Modulates Hippocampal Activation During Memory Encoding in Early Alzheimer's Disease According to Apolipoprotein E-epsilon-4 Genotype

The aim of this experiment was to characterize hippocampal responses to insulin in early AD, and secondarily to assess differences in insulin responses across apolipoprotein E-epsilon-4 genotype in AD. Insulin has been shown to improve memory acutely in early AD, and this phenomenon appears to be most pronounced in apolipoprotein E-epsilon-4 negative AD subjects. The hippocampus is a structure key to memory function, and is known to express insulin receptors that might modulate long-term depression and potentiation mechanisms, suggesting that insulin might act at the hippocampus to influence memory performance. The results demonstrate that hippocampal activation in AD between the saline and insulin conditions differs according to apolipoprotein E-epsilon-4 genotype, with carriers exhibiting decreased hippocampal activation in response to insulin. The changes in hippocampal activation during memory encoding across apolipoprotein E-epsilon-4 genotype were paralleled by changes in image recall performance, suggesting a link between imaging and cognitive testing findings. There were however no insulin-

related changes in performance on the standardized cognitive tests, which might reflect a relatively short duration of action for insulin.

The apolipoprotein E-epsilon-4 based differences in hippocampal insulin responses suggest there might be fundamental differences in insulin signaling between the two groups. Alzheimer's disease subjects who are not carriers of this gene have been shown to have increased risk for type 2 diabetes and obesity, (Profenno & Faraone, 2008) suggesting peripheral insulin signaling alterations in this subgroup of AD subjects, but how this might be related to brain insulin signaling is not clear. Although the hippocampal activation changes between the saline and insulin conditions in the apolipoprotein E-epsilon-4 negative AD group described herein did not reach statistical significance, mean activation did increase in this group with insulin. This increased activity and image recall performance associated with insulin administration might represent preliminary evidence of impaired insulin signaling or perhaps insulin resistance specifically in apolipoprotein E-epsilon-4 negative AD. Furthermore, the observations of attenuated hippocampal responses paralleled by a trend towards decreased cognitive performance with insulin in the apolipoprotein E-epsilon-4 carriers suggest that augmentation of insulin signaling might be detrimental to this AD subgroup, however the possible reasons for this observation are not clear. This general profile of increased cognitive performance in apolipoprotein E-epsilon-4 negative AD, and decreased cognitive performance in

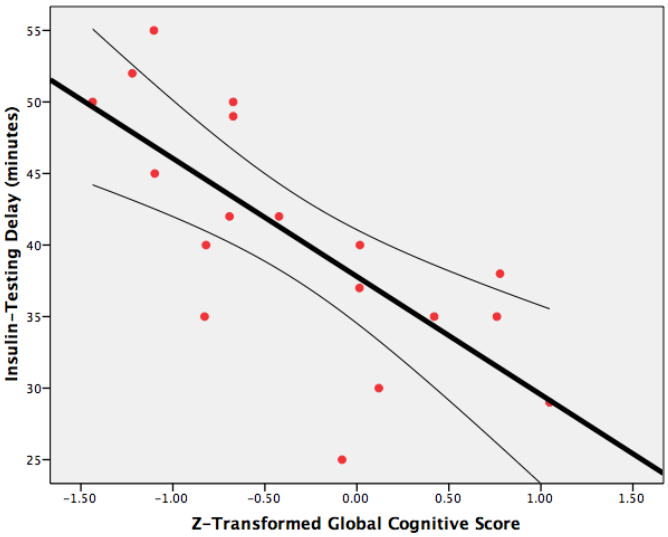
apolipoprotein E-epsilon-4 positive AD with insulin is in line with other reports, and it therefore appears possible that insulin-related changes in hippocampal function might underlie insulin-mediated memory facilitation in early AD.

The lack of insulin-related change in performance on the standardized cognitive testing however suggests an alternate interpretation. It might be that the apolipoprotein E-epsilon-4 positive AD subjects, who showed decreased hippocampal activation with insulin compared to saline along with similar cognitive performance between the saline and insulin conditions, were able to maintain constant cognitive performance with less hippocampal engagement with insulin compared to saline. The findings in the apolipoprotein E-epsilon-4 negative AD group, which exhibited no change in performance on the standardized cognitive testing between the saline and insulin conditions, and demonstrated an increase (although statistically insignificant) in hippocampal activation, might suggest that these subject had to engage more hippocampus with insulin to maintain the performance attained with saline. Thus, in light of the lack of insulin-related change in performance on the standardized cognitive testing, it may not be entirely clear if the changes in hippocampal activation observed are beneficial or even relevant.

It is not clear if more hippocampal activation is necessarily better. Increased BOLD activation for example has been observed in mild cognitive impairment, and might indicate compensatory recruitment of adjacent tissue in a setting of chronic but sub-clinical degeneration. (Borghesani, et al., 2007; Han, et al., 2007) A clear cognitive response to insulin would help clarify if more or less hippocampal activation in response to insulin is actually beneficial in terms of memory. While image recall performance increased with insulin in the apolipoprotein E-epsilon-4 negative AD group in line with previous reports, there were essentially no other definite memory effects by which to judge the hippocampal responses. One possible explanation for this is that the time between insulin administration and testing during this study was much greater than reported by others, (S Craft, S Asthana, G Schellenberg, et al., 1999; Craft, et al., 1996; M. A. Reger & S. Craft, 2006; G. S. Watson & Craft, 2004) where testing began within 15 minutes after insulin administration. The times of insulin administration and the start of the first BOLD fMRI sequence were recorded, (mean delay 41.4 minutes, SD 9.3 minutes) and it was found that there was a negative correlation between this time delay and global cognitive performance, but only in the AD subjects, (Spearman's Rho = -0.791, $p < 0.001$) while no correlation was evident in the AD subjects with saline, or in the control group with either saline or insulin. (Figure 6-1)

Most of the variability in this delay was related to the necessity of placing the subjects in the scanner and adjusting the back projection system, while the pace of the subsequent events preceding the cognitive testing was fairly steady, dictated mostly by the programming of the MRI scanner. There were no differences in the delay between the saline and insulin conditions, (Wilcoxon test, p=0.52) or in this delay between the control and AD subjects. (Mann-Whitney U test, p=0.46) The negative correlation between drug-testing delay and global cognitive performance in AD with insulin might suggest that any effects of insulin were waning by the time of formal cognitive testing. That an insulin-related change in image recall performance was noted might be related to the fact that the timing of this test during the sequence of procedures placed it as the first cognitive measure after insulin administration, although typically no more than 10-15 minutes before the standardized cognitive testing. The CSF pharmacokinetics of insulin delivered via nasal spray are not well documented, but the results of this

Figure 6-1-Relationship Between the Insulin Administration-Cognitive Testing Delay and Cognitive Performance.



Scores on the standardized cognitive testing measures negatively correlated with the delay between insulin administration and testing.

study might suggest that any cognitive effects are short lived, perhaps on the order of an hour or so.

Chapter 4-Insulin and fMRI Motor Activity in Early Alzheimer's Disease

This experiment was conducted to assess insulin's effect on non-memory related tasks as a control for the hippocampal activation experiment. If insulin acts specifically at hippocampal insulin receptors to modulate long-term depression and potentiation mechanisms that drive changes in cognitive performance, then activation during a simple motor task, unrelated to memory encoding, should show no change between the saline and insulin conditions.

The results of this study however demonstrated that insulin might alter task-related activation at the ipsilateral (i.e. right) motor cortex according to apolipoprotein E-epsilon-4 genotype in AD. The motor findings therefore appear to parallel those of the memory encoding experiment, and suggest that insulin might act in a general and non-specific manner, rather than specifically at the hippocampus to modulate brain function in AD.

Chapter 5-Mean Cerebral Blood Flow is Not Altered After Intranasal Insulin Administration

Insulin is known to act as a vasodilator peripherally via an endothelial nitric oxide (NO) dependent mechanism. (Dallinger, et al., 2003; Randriamboavonjy, Schrader, Busse, & Fleming, 2004) Such vasodilatory effects could contribute to insulin effect on memory by increasing delivery of glucose and oxygen to either the entire brain, or regionally to metabolically active areas. Furthermore, pharmacologically induced changes in blood flow can confound BOLD-based imaging. For these reasons, an attempt was made to non-invasively measure mean CBF. Arterial spin-labeled MRI (ASL-MRI) data were acquired on the last 21 participants. (8 controls and 13 early AD subjects) It was anticipated that insulin would increase mean CBF compared to saline. The results of the ASL-MRI experiments showed no statistically significant change in mean CBF between the saline and insulin conditions. There appeared to be however a large degree of variability in the individual measures. Post-hoc calculations showed the 95% confidence interval of the mean change between conditions to be -9.47-11.59 ml/100g/min, again suggesting that there was no insulin-related change in mean CBF. It might be that the ASL method used is not sensitive to small changes in mean CBF, or that in fact insulin did not alter mean cerebral blood flow. The absolute values obtained by this method (saline; 81.8 ml/100g/min, SD 26.8, insulin

82.9, SD 25.9) are however significantly different from the generally accepted mean of 50 ml/100g/min for normal gray matter, (mean CBF with saline, one-sample t-test, $p<0.001$) suggesting that the method used lacked accuracy. Alternatively, insulin might modulate *task-related changes in regional blood flow,* rather than mean cerebral blood flow during rest. Thus, assessment of changes in task-dependent regional cerebral blood flow with insulin might be more informative than resting mean cerebral blood flow.

Insulin and Brain Health and Function in Alzheimer's Disease

The work presented herein was undertaken to characterize the role of insulin in hippocampal structure and function in early Alzheimer's disease. The results of preliminary experiments demonstrated first an AD-specific positive correlation between peripheral insulin levels and hippocampal volume, consistent with insulin's proposed neurotrophic and neuroprotective properties. In terms of memory function, there were distinct differences in the hippocampal and medial temporal lobe fMRI activation with insulin across apolipoprotein E-epsilon-4 genotype in AD. The changes in hippocampal activation were paralleled by changes in cognitive performance suggesting that modulation of hippocampal and related medial temporal lobe activity by insulin could explain reports of insulin-related memory facilitation in AD. The motor fMRI experiment also demonstrated an apolipoprotein E-epsilon-4 based interaction in AD subjects similar to that seen for the memory task. In the context of understanding the general effects of insulin on brain activity, the results of the motor fMRI experiment suggest that insulin might influence extra-hippocampal regions to contribute to cognitive enhancement. Last, mean cerebral blood flow measured with arterial spin-labeled MRI was the same in both the saline and insulin conditions, suggesting that insulin's effects on brain activity are not mediated by changes in mean cerebral blood flow. These experiments suggest an AD-specific role for insulin in maintaining hippocampal volume, and contributing to the growing

body of research suggesting that insulin may play an important role in modulating AD pathology. Insulin also appears to influence neuronal physiology in AD according to apolipoprotein E-epsilon-4 genotype. During both the memory and motor tasks, apolipoprotein E-epsilon-4 negative AD subjects exhibited greater task-related BOLD activation with insulin compared to saline than their apolipoprotein E-epsilon-4 positive counterparts. The fact that insulin's trophic effects on hippocampal volume do not appear to differ according to apolipoprotein E-epsilon-4 genotype however suggest that the long-term and acute effects of insulin might be mediated by different mechanisms. It is hoped that the results of these studies will contribute to the growing body of research focused on brain insulin signaling in AD, and ultimately translate into novel AD prevention and treatment strategies.

Insulin might act to promote neuronal health and survival in several manners. The most obvious is its role in the promotion of oxidative metabolism. Insulin signaling is linked to hexokinase and phosphofructokinase activity, enzymes that initiate glycolysis and therefore ultimately lead to ATP production. Considering the nearly universal role of ATP in practically all cellular functions, impaired insulin signaling might be expected to impact a variety of energy-dependent activities. Insulin dysfunction along with altered glucose metabolism appears to occur in AD. (S. Craft, et al., 2000; Craft, et al., 1993; Frolich, et al., 1999; Hoyer, 2004a, 2004b) It therefore seems possible that insulin signaling abnormalities might contribute to

cerebral metabolic disturbance in AD. Insulin might also act as a specific growth factor, influencing neuronal health and survival. Insulin-like growth factor-1 (IGF-1) shares structural and signaling similarities with insulin, and is known to increase neurogenesis in the adult rat hippocampus, (M. F. Anderson, Aberg, Nilsson, & Eriksson, 2002) and several studies have shown decreased insulin and IGF-1 action specifically in AD.(de la Monte & Wands, 2005; Rivera, et al., 2005; Steen, et al., 2005) It might be that insulin is related to neurogenesis (Jackson-Guilford, Leander, & Nisenbaum, 2000) during normal aging, and that impaired insulin action seen in the AD brain might therefore contribute to neuronal loss.

Insulin also appears to play a central role in AD neuropathology. Abeta-42 is a product of amyloid precursor protein (APP) metabolism and a major constituent of the amyloid plaques seen in AD. The amyloidogenic degradation of APP begins with initial cleavage by beta-secretase, followed by further action on the resulting C-terminal fragment by gamma secretase. Insulin acts to decrease Abeta production by inhibiting gamma-secretase activity via glycogen synthase kinase-3-beta (GKS-3β) inhibition. Thus impaired insulin signaling might remove inhibitory control over gamma-secretase activity and lead to increased Abeta production. (Phiel, et al., 2003) An GSK-3 isoform GSK-3α acts to phosphorylate tau, and is also under inhibitory influence of insulin signaling mechanisms. Impaired insulin action therefore might also promote tau hyperphosphorylation and contribute to neurofibrillary tangle

formation characteristic of AD. (Muyllaert, et al., 2006) These two isoforms of GSK-3 would therefore appear to be central to insulin's effects on AD neuropathology, and the role of GSK-3 in this regard is substantiated by reports of reduced Abeta production after lithium (an inhibitor of GSK-3 activity) administration. (Phiel, et al., 2003) Further evidence of insulin's effect of AD neuropathology is provided by reports of reversible tau phosphorylation in streptozotocin (STZ) treated rats. (Schechter, et al., 2005) In normal humans, Insulin administration is associated with increased CSF Abeta-42, consistent with decreased deposition and increased clearance of this amyloidogenic species. (G. Watson, et al., 2003) Insulin administration also appears to increase the plasma Abeta-40/42 ratio, ((M. Reger, Watson, Green, Wilkinson, et al., 2008) again suggesting that insulin signaling can modulate amyloid metabolism in a favorable manner. Thus, it appears that insulin modulates the metabolism of both Abeta and tau in a manner that inhibits AD neuropathology, possibly by insulin's inhibitory influence on GSK-3 mechanisms, and therefore might modulate AD neuropathologic expression or burden. The results of the voxel-based morphometry experiment in chapter 2, which demonstrated greater hippocampal volume in those AD subjects with higher peripheral insulin levels, would support the notion that insulin acts reduce pathologic burden and decrease neuronal loss at the hippocampus.

The data presented herein describe AD-specific insulin effects on both hippocampal volume and function. The positive correlation between peripheral insulin levels and limbic/hippocampal volume in AD would support a neurotrophic role for insulin. The insulin measure used for this exploratory study, insulin AUC during a three-hour intravenous glucose tolerance test, represents endogenous insulin secretion and production to a standardized glucose challenge, and therefore might be less susceptible to day-to-day variation in fasting insulin levels and better reflect long-term insulin exposure over a variety of physiologic conditions. It is important to note that the participants in this study were non-diabetic by common definitions. Those AD subjects with higher insulin exposure exhibited greater gray matter volume in limbic and hippocampal regions. While it is not clear from these data if insulin acts to increase gray matter volume, or rather slows age or disease-related atrophy in regions known to express insulin receptors, this relationship adds to the growing body of evidence that insulin signaling mechanisms might be important in preserving brain health in AD. An alternate interpretation would suggest that those AD subjects exhibiting relative insulin resistance, reflected by higher insulin AUC during the three-hour intravenous glucose tolerance test, is beneficial in terms of limbic/hippocampal volume. This scenario seems unlikely however when considering the body of evidence indicating that insulin signaling mechanisms are beneficial to neuronal health.

The functional MRI results presented herein suggest that insulin might serve as neuromodulatory role in AD in addition to the proposed neurotrophic or neuroprotective roles. The memory encoding experiments demonstrate that insulin modulates hippocampal activity according to apolipoprotein E-epsilon-4 genotype in early AD, with significant decreases in hippocampal activation in apolipoprotein E-epsilon-4 positive AD. These changes were paralleled by changes in performance on an image recall test, suggesting a link between the imaging and cognitive findings and might indicate that insulin has a specific function in hippocampal-based memory performance.

Insulin might act to modulate hippocampal activity during memory encoding by altering N-methyl-D-aspartic acid (NMDA) receptor function. Insulin signaling mechanisms have been shown to transiently phosphorylate tyrosine residues on NR2A and NR2B subunits in rat hippocampal slices, and increasing NMDA activity. (Christie, et al., 1999) A similar phenomenon has been observed in xenopus oocytes. (Jones & Leonard, 2005) Insulin appears to restore impaired long-term potentiation (LTP) function in rat hippocampal sliced obtained from diabetic rats, (Izumi, et al., 2003) while glucose alone did not restore LTP function, underscoring that insulin might have a specific relationship to LTP mechanisms. Insulin along with low-frequency stimulation promotes phosphorylation of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors at specific GluR2 subunits, leading to

clathrin-mediated endocytosis and long-term depression (LTD) in rat hippocampal CA1 neurons. (Ahmadian, et al., 2004) Insulin-induced changes in these LTP/LTD mechanisms might be expected to alter synaptic activity, modulating BOLD activation as well as cognitive performance. How apolipoprotein E-epsilon-4 genotype however might impact these mechanisms is not clear. It appears therefore that insulin might modulate both LTP and LTD, perhaps in a stimulus-dependent manner, at the hippocampus to facilitate cognitive function in AD.

The results of the motor experiment suggest that insulin might affect non-memory processes according to apolipoprotein E-epsilon-4 genotype in AD, similar to the manner noted during the memory task. While the specific molecular mechanisms underlying these observations cannot be determined from these studies, the similar response to insulin for both the memory and motor tasks would suggest some degree of overlap in the mediators of these effects. As mentioned earlier, insulin administration increases CSF norepinephrine levels and there appear to be apolipoprotein E-epsilon-4 based differences in noradrenergic signaling. It might therefore be that insulin's effects are mediated via noradrenergic signaling mechanisms.

It is interesting to note that there were no apolipoprotein E-epsilon-4 related differences in the relationship between peripheral insulin and limbic/hippocampal volume, while there were clearly differences in hippocampal function according to this AD risk factor. Several potential mechanisms by which insulin might act to influence neuronal health and function have been described, and this disparity would suggest that these two effects are mediated by unique phenomenon.

Brain Insulin Resistance in Alzheimer's Disease

A recent review summarizes the growing body of evidence suggesting the existence of brain insulin resistance in AD. (de la Monte, 2009) In brief, there appear to be reductions in insulin and insulin-like growth factor (IGF) receptors and signaling intermediates that result in impaired glucose utilization similar to that seen peripherally in type 2 diabetes mellitus, prompting the term "type 3 diabetes" to describe AD pathophysiology. Impaired insulin signaling also contributes to aberrant tau phosphorylation and Abeta deposition, as well as oxidative stress. Thus, the alterations in insulin signaling seen in AD might underlie the anatomic and physiologic changes seen in AD.

These observations have been described as evidence of brain insulin resistance. In a setting of insulin resistance for example, higher insulin levels might be able to restore insulin's beneficial effects on neuronal health and inhibitory influence on AD pathology in regions known to exhibit high concentrations of insulin receptors, such as the hippocampus. That we observed a relationship between insulin and hippocampal volume in the AD group suggests that chronic exposure to higher insulin levels might ameliorate any impairment in insulin signaling, and act to preserve hippocampal volume, perhaps by inhibiting AD neuropathology.

We further observed insulin-induced changes in hippocampal function in AD according to apolipoprotein E-epsilon-4 genotype, suggesting first that this common AD risk factor might define two distinct sub-populations with different responses to insulin. In the non-carriers, hippocampal activation showed a non-significant increase in hippocampal activation with insulin, while activation in carriers decreased with insulin. This might be interpreted as preliminary evidence of brain insulin resistance in terms of acute function in the non-carriers. Furthermore, augmentation of insulin action might be detrimental in apolipoprotein E-epsilon-4 AD carriers, although the reasons for this are obscure.

Interestingly, there does not appear to be apolipoprotein E-epsilon-4 related differences in the relationship between insulin and hippocampal volume. This might suggest that the acute and chronic effects of insulin are mediated by unique biochemical pathways. In fact, insulin's trophic effects involve GSK-3 signaling, while insulin's potential effects on cognition likely involve NMDA and AMPA receptors.

The results of the motor control task suggest that insulin might influence non-memory related tasks as well. In the overall AD group, brain activation increased in several regions, including the ipsilateral somatosensory cortex. There also appears to be an apolipoprotein E-epsilon-4 based difference in motor responses similar to that seen during the memory task, suggesting that insulin acts to modulate widespread brain physiology.

Changes in general cortical excitability with insulin might explain the possible similarities in the insulin responses during the memory encoding and motor tasks. Norepinephrine (NE) is known to modulate cortical excitability and causes the inactivation of slow-acting, Ca++ dependent K+ channels in cortical and hippocampal neurons. These K+ channels are responsible for the "undershoot"

phase of the action potential, and decreasing their current attenuates after-hyperpolarization, promoting sustained action potential generation. (Andersen, et al., 2007)

The sole source of brain NE is the locus ceruleus in the pons. Axons from the locus ceruleus project via the medial forebrain bundle to most cortical areas, branching several times such that a single locus ceruleus cell body might modulate thousands of cortical neurons. (Aston-Jones & Cohen, 2005) Norepinephrine acts at cell body autoreceptors within the locus ceruleus, promoting electronic coupling between adjacent locus ceruleus neurons, and coordinated phasic activity. Norepinephrine output to cortex from the locus ceruleus is driven by phasic activity, and contributes to attentiveness and cognitive performance (Usher, Cohen, Servan-Schreiber, Rajkowski, & Aston-Jones, 1999) The effects of norepinephrine on phasic activity at the locus ceruleus are regulated by NE reuptake via NE transporter, (NET) and the effect of NET modulation on both cortical excitability and cognitive performance has been demonstrated with several drugs. A single dose of reboxetine, a selective NE reuptake inhibitor, has been shown to increase cortical excitability as measured with transcranial magnetic stimulation, and improve motor skill learning. (Plewnia, Hoppe, Cohen, & Gerloff, 2004) Modafinil, a monoamine reuptake inhibitor, has been shown to modulate locus ceruleus activity on fMRI, (Minzenberg & Carter, 2008) and is known to impact cognitive performance as well, (Marchant, et

al., 2009; Minzenberg & Carter, 2008; Walsh, Randazzo, Stone, & Schweitzer, 2004) Therefore, modulation of locus ceruleus activity by NET inhibition might translate to increased cortical excitability and improved memory performance.

Insulin appears to promote phasic locus ceruleus activity by modulating NET expression.. Insulin has been shown to inhibit NET mRNA production in rat locus ceruleus (Boyd, Clarke, Muther, & Raizada, 1985) in cell culture and after extended intraventricular injection. (Boyd, et al., 1985; Figlewicz, et al., 1993) It might therefore be that insulin's influence on cognitive performance in AD reflects changes at the locus ceruleus leading to increase NE output to cortex (including hippocampus), resulting in increased cortical excitability, and translating to improved cognitive performance. Changes such as these might partially explain some of the finding of this study, however does not address the AD-specific nature of those findings, or the apolipoprotein E-epsilon-4 related differences in insulin responses.

Degeneration of the locus ceruleus in advanced AD has been well documented (Mann, Yates, & Hawkes, 1983; Zweig, et al., 1988) Postmortem examination has more recently shown that locus ceruleus degeneration occurs in normal aging, but is progressively more advanced in mild cognitive impairment

(MCI) and AD. (Grudzien, et al., 2007) The loss of locus ceruleus neurons is accompanied by loss of neurons in prefrontal cortex, but the rate of loss in these two regions is different, with greater prefrontal neuronal loss compared to that of the locus ceruleus. Furthermore, axons of surviving locus ceruleus neurons undergo sprouting, forming additional synapses. (Szot, et al., 2007) This differential rate of neuronal loss between the locus ceruleus and cortex, coupled with axonal sprouting of locus ceruleus neurons, results in a relative increase in noradrenergic input to cortex in AD. Additionally, AD cortex appears to exhibit increased excitability, possibly due to changes in receptor types. (Di Larzzaro, et al., 2003; Di Larzzaro, et al., 2004) Thus, noradrenergic input from the locus ceruleus to cortex appears to be augmented in AD. If insulin acts to modulate cortical function via noradrenergic mechanisms, these pathologic changes might explain in part the observation of increased BOLD activation with insulin specifically in the AD group, while the control group demonstrated responses with insulin that were the same as with saline.

The apolipoprotein E-epsilon-4 based differences in brain insulin responses may reflect differences in noradrenergic signaling mechanisms. The apolipoprotein E-epsilon-4 genotype appears to interact with b2 polymorphisms to confer increased AD risk. These polymorphisms also exhibit different signaling qualities, (Yu, et al., 2008) suggesting that noradrenergic mechanisms might differ across apolipoprotein E-epsilon-4 genotype. It has also been noted that apolipoprotein E-epsilon-4

genotype might be associated with altered noradrenergic signaling in cognitively intact older adults. The antidepressant mirtazapine, which inhibits the reuptake of both norepinephrine and serotonin, appears to work faster in apolipoprotein E-epsilon-4 positive subjects compared to non-carriers. Interestingly, the selective serotonin reuptake inhibitor paroxetine seemed to have the same efficacy across apolipoprotein E-epsilon-4 genotype in this same study, (Murphy, Kremer, Rodrigues, & Schatzberg, 2003) underscoring the relationship between apolipoprotein E-epsilon-4 and NE signaling. While it is not clear how this might relate specifically to the results of this study, these studies suggest that apolipoprotein E-epsilon-4 based differences in both adrenergic and noradrenergic signaling mechanisms might exist. In the context of the results of this study, the existence of such apolipoprotein E-epsilon-4 based differences in noradrenergic sensitivity might support the theory that insulin acts to modulate cognitive performance and brain responses along apolipoprotein E-epsilon-4 genotype by modulating locus ceruleus activity and influencing cortical excitability via a NE-dependent mechanism.

In summary, insulin appears to exhibit an AD-specific relationship with hippocampal volume, which might reflect neurotrophic or neuroprotective effects, and adds to the growing body of evidence that brain insulin signaling might be altered in AD. Insulin also appears to modulate hippocampal activity during

memory encoding, but insulin's effect on hippocampal function appear to differ across apolipoprotein E-epsilon-4 genotype, with carriers exhibiting decreased activation with insulin. These changes in hippocampal activation with insulin appear to be paralleled by changes in some cognitive measures, suggesting a link between imaging and memory findings. Activation during a motor task also appears to increase with insulin in AD, and might follow a similar pattern across apolipoprotein E-epsilon-4 genotype. Insulin however does not appear to influence mean cerebral blood flow.

These insulin-related changes might be explained in part by the possibility that insulin modulates locus ceruleus activity, and therefore influence cortical excitability. Insulin-induced phasic activity at the locus ceruleus might increase norepinephrine output to norepinephrine-hypersensitive AD cortex. Noradrenergic signaling mechanisms might differ according to apolipoprotein E-epsilon-4 genotype, leading to differential effects both in terms of cognitive and BOLD responses between carriers and non-carriers.

Clinical Implications

Insulin or insulin-based therapies might offer new hope for Alzheimer's disease patients and their families. Insulin has been shown to improve cognitive performance acutely (S Craft, S Asthana, G Schellenberg, et al., 1999; Craft, et al., 1996; Murphy, et al., 2003; M. A. Reger, et al., 2007; G. Watson, et al., 2003) and to might modulate Abeta metabolism as well (M. A. Reger, et al., 2007; G. Watson, et al., 2003) suggesting its use to treat AD clinically. It seems however that insulin itself might not be the best choice for long-term clinical use. Insulin and Abeta are degraded by a common enzyme, insulin-degrading enzyme, and it follows that higher insulin levels might actually promote the accumulation of amyloid due to competitive inhibition of insulin degrading enzyme. Since insulin might also decrease Abeta production, the net effect on Abeta deposition would probably represent a balance between insulin's potential effect of promoting amyloid deposition, and inhibition of Abeta production. It is not clear where this balance however might lie. Although a single dose of experimentally administered insulin appears to be safe, clinical use of insulin therapy for AD might be hazardous since cognitively impaired patients might be at increased risk for accidental overdose and potentially life-threatening hypoglycemia. Rosiglitazone, which acts by up-regulating the transcription of insulin signaling intermediates, is used to increase insulin sensitivity in type 2 diabetes, and has been shown to improve cognitive performance in humans as well as in animal models. (Pedersen, et al., 2006; Risner,

et al., 2006) Such an indirect approach to augmenting insulin's effects might offer a safe alternative to insulin itself, and also circumvents the potential for increased Abeta accumulation with insulin. These potential benefits of drugs such as rosiglitazone must however be weighed against their cardiovascular risks. Furthermore, insulin resistance in type 2 diabetes is modifiable with physical activity and lifestyle changes, and these measures might offer some benefit in AD as well. Higher levels of physical activity have been shown to be associated with less age-related cognitive decline and dementia risk, (Verghese, et al., 2003) in support of this notion and further suggesting a protective influence of insulin. Our results also suggest that apolipoprotein E-epsilon-4 genotype might be an important consideration for future development and application of insulin-based therapies for AD. Thus, measures to improve insulin signaling might have both protective and therapeutic benefits for AD, and apolipoprotein E-epsilon-4 genotype might identify those patients most likely to respond to such treatment.

Limitations

In all of the presented studies, the diagnosis of Alzheimer's disease was made on clinical grounds, which are by nature imperfect. The voxel-based morphometric analysis presented in chapter 2 is cross-sectional in design, which makes the determination of cause and effect relationships impossible. It is therefore not clear if higher insulin levels truly prevent hippocampal volume loss. The white matter results of this study should be interpreted with caution as these might represent misclassified periventricular white matter pathology, although the method used reduces such error. (Good, et al., 2001) Lastly, peripheral insulin was used as a surrogate measure for CNS insulin, and while CNS insulin is derived from the periphery, (Banks, 2004) this may not accurately reflect CNS levels.

The functional MRI studies must be interpreted with some limitation sin mind as well. First, these pilot studies were based on a relatively small sample size, which might limit the ability to detect meaningful changes between saline and insulin. Second, several of the demented subjects were taking drugs such as cholinesterase inhibitors, NMDA agonists and beta-channel-blockers, which might alter fMRI activation patterns. Third, because of the potential that overt insulin resistance could confound the study, we excluded diabetic from participation. This however might bias our results as apolipoprotein E-epsilon-4 AD subjects are at

increased diabetes risk. Forth, we did not measure insulin levels in CSF, and it is possible that variable penetration was achieved with the administration method used. Fifth, the order of event during each visit was dictated such that the fMRI data would be obtained when CSF insulin levels were at their peak after intranasal insulin administration as reported in the literature. Consequently, the cognitive testing occurred after the MRI scan, and it appears that any of insulin's effects might have decreased by that time. The lack of clear cognitive responses to insulin makes it difficult to determine if the changes in hippocampal activation observed are beneficial in terms of cognitive performance.

Future Directions

Degenerative changes within brain noradrenergic systems in AD might be contribute to cognitive impairment in AD, and might represent a novel therapeutic target. Drugs that inhibit norepinephrine reuptake such as modafinil and reboxetine might have been shown to modulate locus ceruleus activity and improve cognitive performance, and therefore deserve study as potential AD treatments. Their effect on hippocampal activation however has not been assessed. Comparing the effect of these drugs, whose mechanism of action is known, to that of insulin might offer insight into insulin's mode action, and could also suggests new AD treatments.

Addendum-Sex Differences in Insulin-Related Changes in fMRI Activation

Several reports suggest sex-based differences in fMRI activation. Garn et al describe sex-by-category interactions during an object naming task, with selective activation of the anterodorsal cingulate and left posterior temporal gyri in females for tools, and left posterior middle temporal gyrus for plants in males. (Garn, Allen, & Larsen, 2009) Interestingly, there appear to be hormonal influences on brain function in both men and women. In addition to several common areas of activation during a semantic retrieval task, men exhibited greater left frontal activation than women in both the early follicular and midluteal menstrual phases. In women, left frontal activation was shown to correlate with estradiol in the midluteal phase and with progesterone during both phases. (Konrad, et al., 2008) It seems therefore that sex might influence the results of fMRI studies, potentially confound imaging studies, but these differences might be less significant in older adults. Few studies comparing the influence of sex on brain activation have however focused specifically in memory related tasks. Schmidt et all studies brain activation during an n-back memory task in normal adults and found no sex-based differences in either response times of brain regions activated during the task. (Schmidt, et al., 2009) These findings are consistent with an earlier report by Haut et al, showing similar activation patterns during working memory and recognition tasks in males and females.

Differences in motor function according to sex have also been described. Left sensorimotor cortex appears to be more involved during non-verbal facial

movements in young men compared to young women.(Fukunaga, et al., 2009) Women showed greater ipsilateral and contralateral motor cortex activation during a finger-tapping task compared to men, while men activated greater subcortical activations compared to females. (Lissek, et al., 2007) In summary, it seems that sex differences during fMRI cognitive tasks might be contextual (i.e. differ according to "maleness" or "femaleness" of stimuli such as tools and plants), and are likely susceptible to hormonal influences. Similarly, there appear to be sex-based differences in fMRI activation during non-cognitive tasks.

We therefore assessed sex-based differences in demographic data as well as fMRI activation during the memory encoding task described in Chapter 3, and the motor task from Chapter 4. Group differences in age, weight, education, fasting glucose and insulin levels, and hippocampal percent activation were assessed with the Kruskall-Wallis test. Differences in plasma glucose and insulin levels between the placebo and insulin conditions were assessed with Wilcoxon Signed-Ranks test. All statistical analysis outside of imaging space was performed with SPSS 16.0. A random-effects model was used to compare brain responses across sex. Voxels exhibiting differences in activation across sex groups were considered significant at $p<0.001$, uncorrected for multiple comparisons.

Control Group-Three male and 7 female control subjects participated in this study. The males and females were similar in terms of age, years of education, weight, baseline fasting glucose, hippocampal percent activation volume and cognitive performance in both the saline and insulin conditions. (Table A-1) Analysis of the memory encoding fMRI data showed no sex-wise differences in medial temporal lobe or hippocampal activation in the saline condition. Comparison of insulin-related change in fMRI activation during the memory encoding across sex however showed greater activation in the males compared to females at the left parahippocampal gyrus (x,y,z = -21, -52, 1) (Figure A-1) Analysis of the motor task in the saline condition data showed greater activation in females at the left post-central gyrus (x, y, x = -39, -40, 58) compared to males. There were however no sex-related differences in insulin related changes in motor activation.

Early AD Group-Thirteen male and 5 female early AD subjects participated in this study. The males and females were similar in terms of age, years of education, baseline fasting glucose, hippocampal percent activation volume and cognitive performance in both the saline and insulin conditions. There was a trend towards greater weight in the males compared to the females. (Table A-2) Analysis of the fMRI data revealed greater activation during the memory encoding task in males compared to females at the right parahippocampal gyrus (x, y, z, = 9, -1, -23) in the saline condition. Males also exhibited greater activation change with insulin

compared to females at the left parahippocampal (x, y, z, = -15, -1, -11) and left lingual gyri. (x, y, z, = -33, -73, -8) During the saline condition motor task, females showed greater activation than males at the right pre-central (x, y, z = 61, -10, 31) and post-central gyri. (x, y, x = 39, -25, 31) Males however exhibited greater insulin-related change in activation than females at the right pre-central gyrus (x, y, z = 60, -31, 31 and 33, -13, 58) and post-central gyrus. (x, y, z = 48, -22, 31)

In summary, cognitively intact males exhibited greater insulin-related increases in fMRI signal than females at the left parahippocampal gyrus during the memory encoding task. Similarly, AD males showed greater insulin related increases in fMRI signal than females at the left parahippocampal and lingual gyri during the memory encoding task. AD males also exhibited greater insulin related increases in fMRI signal than females at the left pre- and post-central gyri during the motor task.

There were however no significant sex-wise differences in the ROI-based assessment of hippocampal activation. This method takes into account intersubject anatomic variation, hippocampal atrophy and image registration errors, and therefore might be a more sensitive measure of brain activity. It is noteworthy that no sex-wise differences in activation were noted in the whole brain analysis at the hippocampus proper, in agreement with the ROI-based results.

The extant literature provides somewhat conflicting information about sex-related differences in fMRI responses. Some reports provide convincing evidence of differences in pre-menopausal females, but how this might relate to an older, post-menopausal population is not clear. Furthermore, some of these differences appear to be contextual, and therefore might reflect more complex psychosocial matters including gender identity, rather than purely sex differences. To the extent possible, the stimulus used for the memory encoding task was neutral in terms of emotional valance and activity content. The motor task employed was largely devoid of any cognitive component. There should therefore be minimal concern that the contexts of theses stimuli underlie the sex-related differences seen. These differences therefore might represent authentic sex-based differences in brain activity, however the small sample size, as well as the significantly different sex proportions between the groups make it difficult to draw any firm conclusions. It is possible that some of the voxels exhibiting sex-wise differences might be driven by confounding issues. A larger sample size with equal sex proportions might help clarify any sex-related differences in insulin effects on cognitive function.

Table A-1. Sex Differences in Control Subjects

	Males (n=3)	**Females (n=7)**	**p-value**
Age (years)	72.3 (9.5)	74.1 (5.4)	0.57
Education (years)	18.3 (2.5)	15.2 (2.3)	0.12
Weight (lbs)	166.3 (25.8)	142.7 (30.2)	0.21
Plasma Glucose at Baseline (mg/dl)	96.0 (4.8)	98.8 (15.3)	0.91
HPAV Saline (%)	21.0 (21.0)	15.6 (14.8)	0.57
HPAV Insulin (%)	28.1 (14.8)	18.6 (13.6)	0.43
Global Cognition Saline	0.0 (1.8)	-0.1 (0.9)	0.57
Global Cognition Insulin	-0.7 (2.2)	0.1 (0.4)	0.73

Table A-2. Sex Differences in Early AD Subjects

	Males (n=13)	**Females (n=5)**	**p-value**
Age (years)	73.4 (7.9)	70.4 (7.7)	0.43
Education (years)	16.1 (4.8)	14.8 (1.9)	0.96
Weight (lbs)	180.5 (21.7)	149.8 (36.1)	0.07
Plasma Glucose at Baseline (mg/dl)	93.3 (7.8)	93.3 (13.7)	0.81
HPAV Saline (%)	14.8 (10.7)	10.6 (6.5)	0.59
HPAV Insulin (%)	9.4 (7.0)	6.2 (3.2)	0.35
Global Cognition Saline	-0.2 (1.1)	0.4 (1.2)	0.35
Global Cognition Insulin	-0.2 (1.2)	0.6 (1.1)	0.15

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