DISSERTATION

# INSULIN AND IGF-I PREVENT BRAIN ATROPHY IN DIABETIC RATS INDEPENDENTLY OF HYPERGLYCEMIA

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PREDRAG ŠERBEDŽIJA ENTITLED INSULIN AND IGF-I PREVENT BRAIN ATROPHY IN DIABETIC RATS INDEPENDENTLY OF HYPERGLYCEMIA BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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**ABSTRACT OF DISSERTATION**

INSULIN AND IGF-I PREVENT BRAIN ATROPHY IN DIABETIC RATS INDEPENDENTLY OF HYPERGLYCEMIA

The causation of brain atrophy associated with dementia in diseases such as diabetes and Alzheimer's Disease (AD) is very poorly understood. There are 20.8 million patients in US with diabetes, 4.5 million with AD, and the incidence of both is rising. Their combined annual treatment cost (direct and indirect) for patients with dementia exceed $148 billion. Reduced insulin and insulin-like growth factor (IGF) signaling is a common biochemical feature in brains of diabetic as well as AD patients. Rodents with knockout of brain neuronal insulin receptor display no change in glucose utilization, neurodegeneration, nor impaired learning/memory. However, IGFs may substitute for insulin or be required for its activity. The literature is virtually silent concerning the role of the widespread insulin receptors in the brain. It is possible that brain atrophy is the consequence of a concomitant decline in IGF as well as insulin levels. The causation of adult brain atrophy is understudied, and identification of the factors that help maintain normal brain mass may provide hints as to the causation of neurodegenerative disorders.

Using the streptozotocin type I diabetic rat model in which both insulin and IGF levels are known to be reduced, it is possible to study the effects of one ligand independently of the other. The purpose of this study was to test the hypothesis that insulin, IGF-I, or their combination can prevent the loss of brain mass, loss of cells, and cell type-specific proteins in the context of diabetes through processes unrelated to glucoregulation. In other words, the hypothesis is that brain atrophy is the consequence

primarily of the loss of two key growth factors in the brain rather than due to hyperglycemia *per se*.

In the first study, either insulin or a combination of insulin and IGF was infused into the brain lateral ventricles of diabetic rats for a period of 12 weeks under conditions that had no effect on hyperglycemia. Our data showed that insulin is a potent regulator of adult brain mass, preventing the loss of brain wet, water, and dry weights independently of persisting hyperglycemia and body weight loss characteristic in diabetes. Although insulin by itself had no significant effect on brain cell loss, the combination of insulin and IGF prevented this loss, as well as loss of total protein content in brain and major cytoskeletal proteins α and β tubulin. Immunoblot data showed that important neuronal and glial proteins were reduced in content in diabetes, and treatments prevented such reductions. Myelin-associated proteolipid protein (PLP) and myelin basic protein (MBP) in oligodendrocytes, and glial fibrillary acidic protein (GFAP) in astrocytes, were all reduced in diabetes. Insulin alone as well as its combination with IGF significantly or completely prevented these reductions. Neuron-specific structural proteins light and medium neurofilaments (NF-L & NF-M), β-III tubulin, the enzyme glutaminase, and synapse-localized vesicular protein syntaxin were also significantly reduced in diabetes. The combination did, but interestingly insulin alone did not prevent all of these reductions. Immunohistochemical staining of brain slices from cortex and hippocampus revealed a visible decrease of the glial markers GFAP and PLP, as well as neuronal NF-M and β-III tubulin. In agreement with the Western blot data, the combination treatment prevented all of these decreases, whereas slices from insulin-treated rats appeared intermediate in intensity between the slices from diabetic and combination-treated rats. This was in contrast to synapse-associated proteins SNAP-25 and PSD-95, where neither insulin nor its combination with IGF exerted a

significant effect on their loss, indicating that these proteins are most likely not under insulin/IGF regulation.

The above study did not reveal whether the effects of the combination were entirely due to IGF-I administration. In the second virtually identical study, the diabetic rat groups were treated either with vehicle, the combination of insulin and IGF-I, or IGF-I alone. The data showed that treatment with IGF-I alone was ineffective at preventing loss of wet, water, and dry weights in diabetes, and it did not have an effect on the reduced relative abundance of GFAP, NF-L, and NF-M, while the combination treatment confirmed the results from the first study.

Taken together, these data clearly show that insulin and the combination of insulin and IGFs can prevent brain atrophy and biochemical pathology by non-glucoregulatory processes in diabetes. The combination-treated animals benefit from a synergy between insulin and IGF-I, where both peptides most likely act through their own respective receptors. The combination treatment had effects greater than insulin or IGF-I alone, and it may do so by preventing a catabolic state in brain that precedes DNA loss. These data may have relevance for clinical encephalopathy observed in physiological states of concomitantly reduced insulin and IGFs, such as diabetes and possibly also in AD.

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## PREFACE

There are presently 4.5 million patients with dementia in the USA, approximately half of all nursing home residents have dementia, and the current total annual cost of care (direct and indirect) is greater than $148 billion. Worldwide, there are an estimated 36 million dementia patients. These numbers are projected to triple by year 2050, which will probably seriously impact the capacity to provide quality health care. The tripling may come sooner than anticipated, because diabetes is now recognized as a separate risk factor for dementia independently of cerebrovascular disease, and there is a disturbing epidemic in diabetes. Current drugs do not significantly slow the progressive brain degeneration, and new treatments to slow or arrest disease progression are urgently needed. Brain atrophy with degeneration underlies disease progression, and an improved understanding of its pathogenesis is urgently sought after as a basis for the design of improved therapeutic interventions since very little is known of the factors that regulate adult brain mass.

The main characteristic of diabetes is a chronic and systemic reduction in insulin signaling, which results in hyperglycemia, generation of advanced glycation end-products, microvascular disease, inflammation, oxidative stress, hyperlipidemia, and other metabolic perturbations. While these factors may contribute to the pathogenesis and may further exacerbate brain atrophy and dementia in diabetes, the purpose of the present study is to test the hypothesis that loss of insulin's non-glucoregulatory activity together with the structurally related insulin-like growth factors (IGFs) are major contributors to brain atrophy observed in diabetes. Therefore, the aim of this thesis was 1) to determine whether insulin, IGF-I, or their combination are regulators of adult brain mass, particularly in the context of diabetes, and 2) to test whether brain atrophy and

biochemical disturbances in the brain in diabetes can be ameliorated or prevented by above treatments despite persisting hyperglycemia.

To lay the theoretical foundation for the work that will be described herein, the reader will first be provided with a brief summary of brain atrophy, dementia, and electrophysiological and cognitive disturbances among diabetic patients. Clinical information, however, is limited, particularly in regard to abnormalities in the biochemistry of brain cells in diabetes. The streptozotocin (STZ) diabetic rat will be discussed, because it is by far the most studied model of type I diabetes that simulates the clinical condition of brain atrophy, biochemical, and cognitive impairments that are accompanied by a concomitant reduction in insulin as well as insulin-like growth factor (IGF) signaling. It is the model that we selected to test the above hypotheses, since effects of either insulin or IGF in the brain can be studied independently of the other. Currently available literature regarding regional and cell type-specific brain degeneration and cell death in this model will be reviewed in the Discussion, as well as outcomes of insulin/IGF replacement therapies that did *not* maintain hyperglycemia constant as a variable.

Hyperglycemia and its secondary consequences are widely regarded in the medical literature as the leading culprits for the pathogenesis of diabetic complications, and this literature will be introduced. This will be followed by a presentation of our alternative hypothesis of insulin and IGF-related deficiency as the underlying cause of diabetic encephalopathy. In order to more fully appreciate this alternative hypothesis, the roles of insulin and the structurally related IGFs in the brain will be reviewed, primarily *in vivo* studies that pertain to their trophic effects and their roles as mediators of learning and memory. An extensive search of the PubMed and world-wide web databases reveals a surprising lack of information concerning the neurobiological role of insulin in the brain other than in regulating appetite and satiety.

The tests of our alternative hypotheses are presented in the Results section. These data support the interpretation that insulin and IGFs are regulators of the predominance of adult brain mass. Evidence is provided that these actions of insulin are separate and independent from those involved in glucose regulation. It will be discussed that a complex interplay between insulin and IGFs exists where insulin and IGF may potentiate one another's actions. This may be mediated in part through the sharing of a common intracellular signaling pathway, and even the formation of hybrid receptors. The implication of these findings to brain atrophy in clinical diabetes will be a topic in the Discussion section, including the active regulation of cerebral water content by insulin and its potential relationship to the development of cerebral edema during aggressive insulin therapy in episodes of diabetic ketoacidosis. This thesis will close with a discussion of the strong association between a mid-life history of diabetes and late-onset Alzheimer's disease (LOAD), controversy regarding whether the neuritic plaques and neurofibrillary tangles are pathogenic for dementia in LOAD, and the suggestion that the concomitantly diminished insulin and IGF signaling may be pathogenic for brain atrophy in LOAD as well as in diabetes. In this discussion, we propose that the association between diabetes and LOAD has nothing to do with diabetes per se, e.g. unrelated to hyperglycemia, but rather the association concerns the shared concomitantly diminished insulin and IGF signaling in brain.

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# PART 1. INTRODUCTION

## 1.1. OVERVIEW OF DIABETES

Definition. Diabetes results from a chronic reduction in insulin signaling. There are two types of diabetes: type I and type II. Type I, formerly known as insulin-dependent diabetes mellitus (IDDM), is characterized by a relatively sudden autoimmune response against the host's own insulin-producing β-pancreatic islets of Langerhans. Type I diabetes has been traditionally termed juvenile-onset diabetes due to high incidence of the disease among children. However, since adults have also been documented to develop the disease, that description has fallen out of favor. Type I diabetic patients are under a strict dietary regimen and require regular insulin shots in order to survive. As a consequence, type I diabetics tend to experience hypoinsulinemia and hyperglycemia.

Type II diabetes, formerly known as non-insulin-dependent diabetes mellitus (NIDDM) is by far more common, accounting for more than 90% of all cases. Rather than loss of endogenous insulin production, type II diabetics have either normal or elevated plasma insulin, yet they are resistant to its biological effects. Consequently, they tend to be hyperinsulinemic and hyperglycemic, a condition which can be ameliorated with a regulated diet, exercise, and administration of insulin or insulin-sensitizing drugs such as metformin. Thus, although the causation of impaired insulin-mediated response is different in type I and type II diabetes, their shared pathology is a chronic and systemic reduction in insulin-mediated signaling.

Prevalence, Cost, and Risk Factors. According to the American Diabetes Association, there are currently 23.6 million people in the United States, or 8% of the population, who have diabetes. In 2007 alone 1.6 million new cases were diagnosed in

people 20 years or older and the prevalence is sharply on the rise. If present trends continue, *one in three* Americans born in 2000 are likely to develop diabetes in their lifetime. The actual prevalence figure can be deceiving, since as many as 24% of cases remain undiagnosed, in part because of complications being erroneously attributed as consequences of other diseases. The total annual economic cost of diabetes in 2007 was estimated at $174 billion. If left unchecked, the inevitable increase in expenses could likely overwhelm the health care system. There are many factors that contribute to development of both types of diabetes. While some are believed to increase the risk more than others, consensus largely exists that these factors include age, diet, obesity, family history of diabetes, and lack of physical activity.

Complications Associated with Diabetes. Diabetes is a disease that develops insidiously, but which greatly impacts one's quality of life. Long-lasting diabetic condition multiplies the risk for health complications such as heart disease, stroke, hypertension, blindness, kidney disease, dental disease, complications in pregnancy, sexual dysfunction, and both peripheral and central nervous system damage. The systemic scope and severity of resulting complications helps explain why diabetes is the fifth-deadliest disease in the United States. Based on death certificate reports, diabetes contributed to 233,619 deaths in United States in 2005. Since the disease tends to be under-reported on death certificates, particularly in the cases of older persons with multiple chronic conditions, the impact of diabetes on mortality may be far greater.

Peripheral Neuropathy. About 60% to 70% of people with diabetes have mild to severe forms of peripheral nervous system damage. The results of such damage include impaired sensation or pain in the extremities (fingers, toes, feet), slowed digestion of food in the stomach, and/or carpal tunnel syndrome. Severe cases of diabetic nerve disease are a major contributing cause of lower-extremity amputations. Indeed, the rate of amputation for people with diabetes is 10 times greater than for

people without the disease. In 2004, about 71,000 non-traumatic lower-limb amputations were performed on diabetic patients in the USA. Despite best efforts to keep patient's glycemic levels under control, neuropathy continues in approximately 40% of patients (Diabetes Control and Complications Trial, (1996)).

Complications Associated with the CNS. Even though learning and memory impairments among diabetic patients have been documented since the early 1920s (Miles and Root, 1922), impaired cognition and dementia have been until recently attributed to secondary consequences of aging or as a result of other diseases rather than diabetes itself. The next section will address clinical findings that show diabetes significantly increases the risk for cognitive impairments and dementia among both type I and type II diabetics.

## 1.2. BRAIN ATROPHY AND DEGENERATION IN CLINICAL DIABETES

Postmortem Studies. Brain atrophy and structural lesions are observed in both type 1 and 2 diabetes. Pioneering autopsy studies demonstrated structural lesions (Reske-Nielsen and Lundbaek, 1963) and loss of brain myelin (Palo et al., 1977) in brains from diabetic patients compared to age-matched non-diabetic subjects. However, these studies did not receive wide recognition because of the belief that such lesions were secondary to chronic debilitation. Further postmortem examinations of type I diabetic patient brains revealed that the neuropathological changes affected multiple brain regions including the brain stem, thalamus, globus pallidus, and cerebellum (Patrick and Campbell, 1990).

MRI Studies. Brain atrophy has been detected using magnetic resonance imaging (MRI). Several studies document brain atrophy in long duration type I diabetic

patients, showing sub-cortical and brain stem lesions (Dejgaard et al., 1991), as well as "high-intensity rounded lesions and cortical atrophy, suggestive of premature aging of the brain" (Perros et al., 1997). As many as 70% of young type I patients are reported to exhibit morphological alterations of the brain characterized by variable dilatation of the lateral ventricles and subarachnoidal spaces, indicative of brain atrophy (Lunetta et al., 1994). Likewise, MRI studies involving type II diabetic patients demonstrate brain atrophy to be more frequent compared to age-matched non-diabetic subjects, particularly in the $6^{th}$ and $8^{th}$ decades of life (Araki et al., 1994). Interestingly, these findings were made independently of vascular disease, suggesting an alternate causation. Computerized tomography (CT) scans of brains from type II diabetics also show brain atrophy (Soininen et al., 1992). While it remains ambiguous from MRI scans whether brain atrophy in diabetes is due to loss of brain water or significant loss of brain cells and their biochemical constituents, previously stated autopsy studies clearly show brain lesions, indicating that brain atrophy involves brain cell loss. Although there are conflicting results, these alterations seem to be independent of hyperglycemia and hypoglycemic episodes. Brain atrophy and degeneration can lead to electrophysiological, cognitive and other behavioral disturbances in the brain. These are introduced next.

## 1.3. ELECTROPHYSIOLOGICAL AND COGNITIVE DISTURBANCES IN CLINICAL DIABETES

Electrophysiological Disturbances. Patients suffering from both type I and type II diabetes exhibit electrophysiological disturbances which can occur early in the progression of the disease. Compared to age- and sex-matched non-diabetic control subjects, electrophysiological abnormalities were found in latencies of visual, somatosensory, and brain stem auditory evoked potentials among both type I and type II diabetic patients where the duration of the disease was less than four years and where no concomitant clinical complications were present (Lingenfelser et al., 1993). Delayed auditory brainstem responses were confirmed by others (Donald et al., 1981), consistent with a reduction in saltatory conduction velocity in early clinical diabetes (Pozzessere et al., 1988). Brain stem auditory evoked potentials were also reported to be reduced among type I patients with long duration diabetes (Dejgaard et al., 1991). For a more comprehensive review of electrophysiological abnormalities in clinical diabetes, please refer to a review article by (Brands et al., 2004).

Cognitive disturbances and dementia are observed in both type I and type II diabetic patients. The earliest documented account of impaired memory, mental arithmetic, and psychomotor deficiency in diabetic patients was presented in the early 1920s (Miles and Root, 1922). To date, a number of studies involving both type I and type II diabetic patients implicates diabetes as a risk factor for cognitive impairments and even dementia.

Cognitive Disturbances and Dementia in Type I Diabetes. An evaluation of type I diabetic children aged 6-16 years for intellectual and reading skills indicated that children with early onset and long duration diabetes evidenced greater impairments compared to age-matched non-diabetic subjects (Holmes and Richman, 1985). An auditory verbal

learning test administered to nearly one hundred type I-diabetic children and age- and demographics-matched controls over a 4-year span demonstrated that children with diabetes exhibit poorer verbal mastery and reach a vocabulary plateau where longer disease duration predicted poorer learning over time (Fox et al., 2003). Type I diabetic patients also perform worse than age-matched controls with respect to memory, abstract reasoning, and hand-eye coordination (Franceschi et al., 1984). In tests requiring sustained attention, rapid analysis of visuospatial detail, and hand-eye coordination, type I diabetic patients were found to perform significantly worse despite hypoglycemic episode history, leading the authors to conclude that type I diabetes represents an increased risk for dementia (Ryan and Williams, 1993).

In addition to learning and memory deficits, the incidence of psychiatric conditions such as anxiety, depression, and antisocial personality disorder is increased in type I diabetes (Anderson et al., 2001; Gavard et al., 1993; Lustman et al., 1988; Peyrot and Rubin, 1997; Popkin et al., 1988; Roy et al., 1994). Both anxiety and depression are known to have a negative effect on cognition, and the reported doubling incidence of depression among the diabetic population may contribute to cognitive abnormalities.

Cognitive Disturbances and Dementia in Type II Diabetes. Learning impairments are also observed among type II diabetic patients (Helkala et al., 1995; Ryan and Geckle, 2000). Tasks requiring storage and retrieval of new information was reported to be poorer among type II diabetic patients compared to age-matched controls, although less demanding cognitive tasks such as recall of immediate memory did not seem to be affected (Tun et al., 1990). Extensive literature database review on cognitive impairments among type II diabetic patients revealed that type II diabetic subjects exhibit less effective attention-concentration-working memory when faced with mathematical tasks, slightly worse constructional abilities (determined by object assembly and design), decreased verbal fluency, psychomotor speed, mental flexibility, and abstract reasoning

compared to controls (Stewart and Liolitsa, 1999). Measures of verbal learning, abstract reasoning, and complex psychomotor functioning were also found by others to be worse in type II diabetic than non-diabetic subjects (Perlmuter et al., 1984; Reaven et al., 1990), suggesting memory retrieval difficulties. In a study balanced for age, sex, hypertension, and level of education, impairments in attention, executive functioning, information processing, and memory in type II diabetic patients were found to be associated with brain atrophy as determined by MRI white matter lesions (Manschot et al., 2006). The evidence presented in works cited herein suggests that cognitive impairments in type II diabetes are reminiscent of accelerated aging, which may predispose for dementia. Indeed, population-based studies involving hundreds of participants demonstrate that type II diabetics are at greater risk for dementia compared to age-matched controls (Leibson et al., 1997; Peila et al., 2002). Perhaps the most convincing evidence for type II diabetes being an independent risk factor for dementia has been provided by a prospective, cross-sectional population-based study involving over 6300 elderly subjects in the Rotterdam Study (Ott et al., 1999). The findings are that there is a near doubling of the risk for dementia among type II diabetics after adjusting for age, sex, cerebrovascular disease, hypertension and other confounders (Fig. 1.3.1).

Despite the theory that cognitive impairments arise secondary to glycemic imbalance, there is evidence that shows no impact of glycemic control on impaired

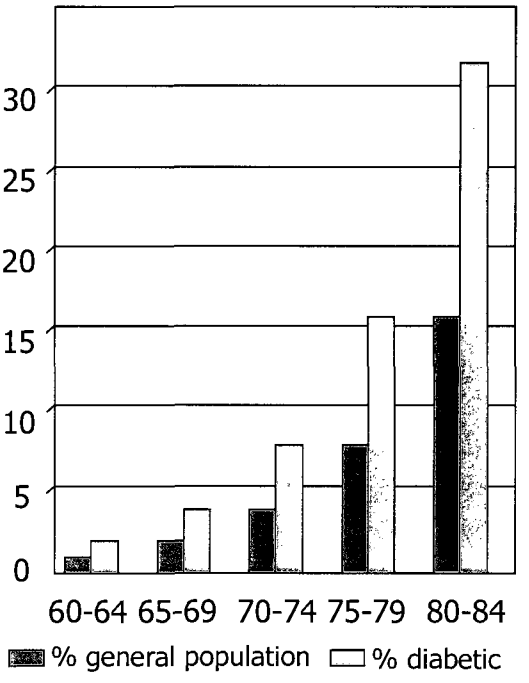



**Fig. 1.3.1.** Risk for dementia (% chance) among type II diabetic and non-diabetic population by age group. Ott et al., 1999.

cognition in diabetes (Prescott et al., 1990). In a relatively large study group, cognitive function in type I diabetic patients was found not to be affected by hyperglycemia (Draelos et al., 1995). Similarly, repeated episodes of severe hypoglycemia affected neither cognitive ability assessed by a neuropsychological test nor brain structure as shown by MRI in young type I diabetic patients (Ferguson et al., 2003).

In summary, brain atrophy in both types of diabetes is associated with electrophysiological, behavioral, and cognitive impairments. Cognitive impairments are progressive with disease duration and aging, and there is elevated risk for dementia in diabetes. Elderly diabetic patients are particularly at risk and cognitive deficits are correlated with brain atrophy on MRI scans.

In order to better understand the underlying pathogenesis of diabetic complications, progression of the disease, and in pursuit of potential treatments, multiple animal models of diabetes are available for experimental studies (reviewed in (Gottlieb et al., 1988; Islam and Loots du, 2009)). One widely used and readily available model is the streptozotocin (STZ)-diabetic rat, which resembles the pathology observed in clinical diabetes. The STZ-diabetic rat was used in this study and will be introduced next. An in-depth discussion of pathological findings in this model with particular regard to results from this study will be presented in the Discussion part of the Dissertation.

## 1.4. THE STREPTOZOTOCIN-DIABETIC RAT MODEL

Shared Pathology Between STZ Rodent Models and Clinical Diabetes. STZ-induced type I diabetes is a suitable model of diabetes that closely resembles the clinical condition. Much like diabetic patients, STZ rats and mice exhibit brain atrophy, degeneration, and loss of brain mass (Francis et al., 2008; Jakobsen et al., 1987; Lupien

et al., 2006). A plethora of literature indicates that degeneration in this model includes morphological and cellular changes in virtually all brain regions (see Discussion). It is affecting many different cell populations, including neurons (Magarinos and McEwen, 2000; Martinez-Tellez et al., 2005; Nitta et al., 2002), astrocytes (Coleman et al., 2004; Lechuga-Sancho et al., 2006), oligodendrocytes (Dheen et al., 1994a), as well as microglia (Luo et al., 2002). There is evidence of impaired synaptic plasticity, diminished myelination, reduced neuritic arborization, and increased cell death.

Brain atrophy and degeneration are accompanied by electrophysiological disturbances in STZ models, resembling the clinical situation. There are impairments in both long-term potentiation (LTP) and long-term depression (LTD), particularly in the hippocampi of these animals (Kamal et al., 1999). LTP and LTD are believed to underlie learning and memory. Indeed, STZ-diabetic animals perform significantly worse on learning and memory tests compared to non-diabetic littermates and age exacerbates that deficit in diabetic animals (Kamal et al., 1999; Lupien et al., 2003). Others have reported disturbances in neurotransmitter levels as well as molecular mechanisms widely believed to underlie learning and memory, while insulin treatments preventing hyperglycemia in STZ rodents showed promise in preventing or reversing such changes. This is further addressed in the Discussion chapter.

Mechanism of STZ-Diabetes. Streptozotocin (STZ) is a glucose analog which is selectively taken up by β-pancreatic cells via GLUT-2 transporters (Szkudelski, 2001). Its cytotoxicity is believed to be primarily the result of alkylation and thus damage to DNA. In turn, DNA damage induces activation of poly ADP-ribosylation, which leads to depletion of cellular $NAD^+$ and ATP stores. This dephosphorylation of ATP is believed to negatively impact oxidative phosphorylation pathways and generate reactive oxygen species. In addition, STZ liberates toxic amounts of nitric oxide. Even though short-lived, nitric oxide can inhibit the activity of the aconitase, an enzyme which participates in

the TCA cycle. As a result, β cells undergo cell death via necrosis and STZ-treated animals develop systemic hyperglycemia within 24 hours due to severe depletion of endogenous insulin production (see Results, p. 79).

Depending on dose of STZ and inherent individual variability between rats, endogenous insulin production is depleted to a different degree. Since insulin is anabolic, these animals essentially cease gaining weight despite a strong appetite and increased water consumption. Most STZ-diabetic rats can survive for several months, although the mortality rate is vastly increased compared to non-diabetic littermates. As a consequence, some investigators resort to a basal insulin treatment regimen in order to keep the animals alive where prolonged duration of diabetes is desired. In our study, no exogenous insulin was provided over the entire duration of 12 weeks, other than the intracerebroventricular (i.c.v.) treatment that did not affect glycemic levels (Materials and Methods p. 38, Results p. 79). Administering a tiny dose of insulin i.c.v. while holding hyperglycemia constant allowed us to discriminate between glucoregulatory and non-glucoregulatory biological effects of insulin on the adult mammalian brain. Due to severe hyperglycemia, STZ animals experience a number of complications that are believed to be the primary culprit for neurodegeneration and learning and memory impairments in diabetes. This "classic" hyperglycemia theory is addressed in the next section, prior to this study's proposed alternative hypothesis.

Taken together, the STZ diabetic rats show brain atrophy, cognitive and electrophysiological disturbances similar to those observed in clinical diabetes. The functional, morphologic, and biochemical abnormalities uncovered in experimental studies support the general regard that the degeneration observed in these animals is a reasonable model of brain pathology observed in the clinical disease.

## 1.5. THE "CLASSIC" THEORY FOR THE PATHOGENESIS OF DIABETIC ENCEPHALOPATHY

Progressive brain atrophy and degeneration most likely underlies disease progression to dementia. However, the pathogenesis of brain atrophy in diabetes remains unclear. This section briefly outlines the "classic" theory centering on hyperglycemia as the primary culprit that has been advanced as the main contributor to pathogenesis of the diverse complications observed in diabetes. For a more thorough review of this theory, the reader is referred to (Brands et al., 2004; Duby et al., 2004).

The theory states that hyperglycemia that accompanies both type I and type II diabetes results in pathogenic processes such as generation of advanced glycation end products (AGEs), oxidative stress, ketoacidosis, osmotic stress, inflammation, and perturbations in cellular biochemical homeostasis. These processes are interconnected and promote one another, exacerbating the disease. This theory is diagrammed in Figure 1.4.1.

AGEs. Generation of advanced glycation end products involves several steps. The aldehyde group of a glucose molecule combines with the amino group of a lysine or arginine residue on a protein to form a Schiff base, a process known as the Maillard reaction. Intramolecular re-arrangement of the Schiff base creates the Amadori product which can be oxidized to form an AGE. Such abnormal non-enzymatic glycation of proteins and other cellular constituents is believed to alter the molecules both structurally and functionally, consequences of which are believed to be cytotoxic.

Flux through the Polyol Pathway. Excess glucose can be reduced by aldose reductase with the oxidation of NADPH to $NADP^+$, producing sorbitol. Sorbitol is further oxidized to fructose by sorbitol dehydrogenase, which is coupled with the reduction of $NAD^+$ to NADH. Sorbitol and fructose are polyols. The high rate of "flux" of glucose

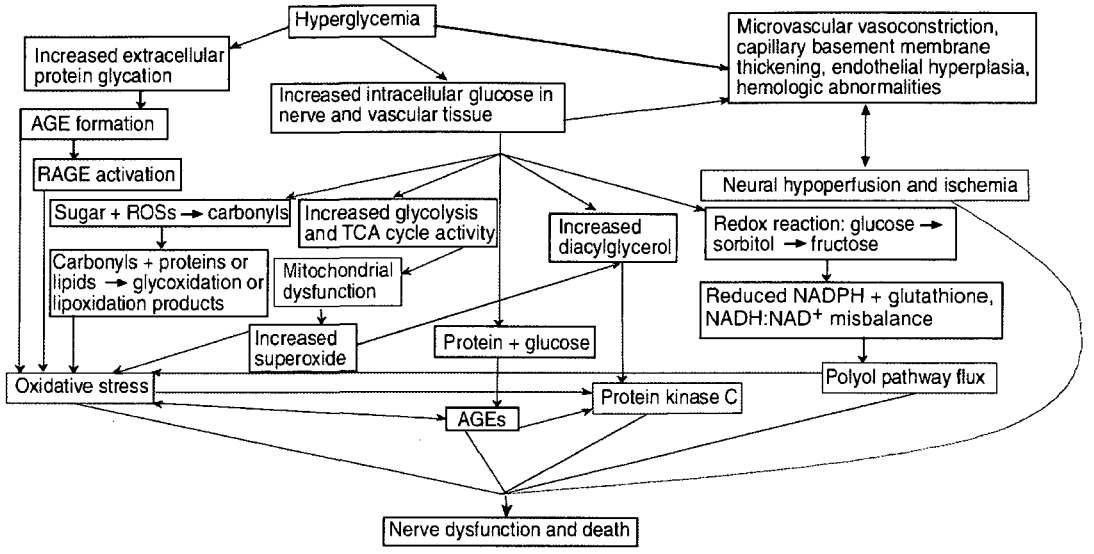



**Fig. 1.4.1.** schematic representation of the "classic" pathogenesis of diabetic complications centering around hyperglycemia and its secondary consequences. Figure from Duby et al., 2004.

through the polyol pathway is believed to be pathogenic, primarily by decreasing NADPH and $NAD^+$, necessary co-factors in redox reactions throughout the body. The decreased concentration of these co-factors leads in turn to decreased synthesis of reduced glutathione, nitric oxide, myo-inositol, and taurine. Generation of fructose, a sugar several times more potent in glycation reactions, and depletion of vital molecular constituents of cells results in a biochemical imbalance that contributes to mitochondrial dysfunction, oxidative stress, and impairments in PKC-mediated signaling.

Secondary Consequences of Hyperglycemia. High availability of glucose is postulated to also lead to a dangerous imbalance in the mitochondrial electron transport chain that could accelerate the production of superoxide and other reactive oxygen species. Due to lack of insulin, there is an increased concentration of ketone bodies in the blood stream, which results in lowering of the blood pH and ketoacidosis. Changes in the microvasculature such as vasoconstriction and capillary basement membrane thickening are also believed to be secondary to hyperglycemia, which may contribute to hypoxia.

It is of considerable interest that treatments with aldose reductase inhibitors, AGE inhibitors, and antioxidants have been shown by clinical trial to be of virtually no benefit to diabetic patients, yet the emphasis on management of diabetic complications including neuropathy remains glycemic control (Duby et al., 2004). While all discussed secondary consequences of hyperglycemia are likely to contribute to and further exacerbate complications associated with diabetes, this work is focused on the roles of insulin in the brain other than in regulation of glucose homeostasis. Indeed, very little is known about the roles of insulin in the adult mammalian brain other than in regulation of feeding and satiety. An in-depth review of the current literature with regard to insulin's non-glucoregulatory roles in the mammalian brain is presented in the next section.

This study is focused on an alternative model of pathogenesis. Recent data show that impaired learning/memory is not due to hyperglycemia and its consequences in diabetes (Lupien et al., 2003). Likewise, brain atrophy is potentially not due to hyperglycemia and its consequences (Lupien et al., 2006). In this section, clinical and experimental data are presented to show that there is a concomitant decline in insulin and IGF signaling in diabetes. This is an important cornerstone for the development of our alternative hypothesis for pathogenesis of brain atrophy which is presented below.

## 1.6. BRAIN INSULIN IS REDUCED IN DIABETES

Sources of Cerebral Insulin. Brain insulin is primarily derived from the circulation by transport across the blood-brain-barrier (Steffens et al., 1988; Woods et al., 2003). That transport is saturable, indicating a controlled, receptor-mediated uptake process. Since insulin receptors (IR) are highly concentrated at the choroid plexus and capillary endothelial cells throughout the brain, the uptake process may be mediated by the IR itself (Baura et al., 1993; Woods et al., 2003). Although there are several reports of local insulin expression in the brain (Devaskar et al., 1993; Steen et al., 2005; Young, 1986), that expression is modest and it is not believed to substantially contribute to total brain insulin concentration. Since insulin was found to be secreted exclusively by neurons (Devaskar et al., 1994; Schechter et al., 1988), a possibility exists that brain-produced insulin may be acting in autocrine or paracrine fashion.

Brain Insulin Levels are Reduced in Clinical Diabetes. In type I diabetes, availability of insulin in the brain is diminished due to reduced systemic availability of insulin. Type II diabetes, on the other hand, is characterized by insulin resistance. Insulin resistance is widespread in older adults and affects approximately half of all adults over age 60 (Craft, 2006). Obesity is a major risk factor for diabetes and low cerebrospinal fluid (CSF) insulin levels are found in obese as well as diabetic subjects (Kern et al., 2006). Insulin resistance is believed to be the cause of reduced uptake into CSF.

Brain Insulin is Reduced in Experimental Diabetes. There are multiple reports of reduced insulin levels in STZ-diabetic rodents. Depending on dose of STZ administration, circulating insulin levels can drop between 40% (Kim et al., 2006) to more than 80% (Arroba et al., 2007). In both clinical as well as STZ models of diabetes, insulin concentration can fall below fasting circulating levels of 1 nM (Ishii, 1995).

Studies investigating binding of radiolabeled insulin to isolated brain microvessels and solubilized brain tissues from control and STZ-diabetic rats produced conflicting data where binding was either diminished (Frank et al., 1986) or unaltered (Pezzino et al., 1996). Nevertheless, in STZ mice, phosphorylation of the IR as well as IR downstream targets Akt and GSK-3β was found to be reduced in brain (Jolivalt et al., 2008). These data indicate that while there may not be down-regulation of the IR in brain in the STZ rodent model of diabetes, there is chronic reduction in insulin availability and IR-mediated intracellular signaling.

In summary, brain insulin signaling is reduced in both clinical diabetes and experimental STZ rodent models of diabetes. The resulting loss of insulin-mediated activity in the brain is proposed to partially contribute to brain atrophy and degeneration in clinical and experimental diabetes. The uniqueness of the brain IR supports that hypothesis.

## 1.7. BRAIN INSULIN RECEPTOR DIFFERS FROM ITS PERIPHERAL COUNTERPART

For a long time the central nervous system was thought to be an insulin-insensitive tissue until a series of experiments performed in the late 1970s demonstrated that insulin and insulin receptors (IR) are present throughout the brain (Havrankova et al., 1978). Ever since that discovery the question concerning the role of the widespread IR has remained largely unanswered. Insulin does not alter glucose utilization in most brain regions within its physiological range of concentrations, although some discrete brain regions such as the hypothalamus are an exception. This section will present data

that show roles of insulin in the brain other than in glucose utilization or regulation of feeding and satiety.

Distribution of IR in the brain. *In situ* hybridization reveals a ubiquitous distribution of IR mRNA throughout the brain, most prominently in the choroid plexus, olfactory bulb, piryform and cerebellar cortices, hippocampus, and hypothalamus (Zhao et al., 1999). $^{125}$I-insulin binding assays on membranes isolated from specific areas and IR-specific immunohistochemistry on slices from rat brain show that IRs are present in most brain regions, with the exception of the basal ganglia and the thalamus (Baskin et al., 1986; Unger et al., 1989; Zahniser et al., 1984).

There is a relatively high density of binding sites throughout development in the olfactory bulb, cortex, hypothalamus, choroid plexus, and cerebellum, while other sites such as brainstem nuclei exhibit high IR presence only during certain periods in early stages (Kar et al., 1993). Maximal binding of radiolabeled insulin in brain slices occurs during the first week of postnatal development and decreases thereafter through adulthood, which correlates with the brain insulin concentration peak during late fetal and early neonatal periods (Schechter et al., 1992).

In adulthood, relatively high density of insulin binding was found in the olfactory bulb, various cortical and hippocampal areas, and the choroid plexus. Thalamus, caudate putamen, and some mesencephalic and brainstem nuclei showed a more diffuse pattern of labeling with moderate to low density of sites. This binding profile correlates closely with reported distribution of IR mRNA. IR densities are higher on neurons compared to glia (Gerozissis, 2003), particularly in synaptic areas of the hippocampus and hypothalamus (Marks et al., 1988; Schwartz et al., 1992).

Structure of Brain IRs. Both α and β subunits of the IR are produced from a common precursor polypeptide which undergoes disulfide bond formation, proteolytic cleavage, and glycosylation, to form a mature dimeric receptor on the cell surface (Olson

et al., 1988). The structure of the brain neuronal and glial IR is somewhat different from its peripheral counterpart. Although both receptors are transmembrane heterotetramers, in addition to two ~94 kDa β-subunits linked by disulfide bonds the brain IR is composed of two 115 kDa α subunits rather than 130 kDa in periphery (Gammeltoft et al., 1985). The neuronal insulin β subunit is also slightly smaller in the brain compared to periphery, while both α and β subunits from brain glial cell cultures are intermediate in size, as determined by SDS-PAGE (Lowe et al., 1986). Subsequent studies found that the difference in size lies in glycosylation of the peripheral and central IR α subunit (Heidenreich and Brandenburg, 1986).

The α subunit is hydrophilic and extracellular, while the β subunit contains more hydrophobic residues representing the single transmembrane region, as well as a longer cytoplasmic tyrosine kinase domain. $K_D$ of insulin binding to its receptor is about 1 nM, indicating high affinity. The $ED_{50}$ of the brain IR (concentration of ligand necessary to elicit half-maximal response) was found to be as low as 2-5 nM, indicating maximal activation of the receptor with physiological concentrations of insulin (Gammeltoft et al., 1985; Gammeltoft and Van Obberghen, 1986). Upon insulin binding, tyrosine kinase activity associated with the β subunit is increased about 3 orders of magnitude (Nakae et al., 2001). Binding of insulin also leads to internalization of insulin-IR complex. Although there is no apparent difference in affinity of insulin for either central or peripheral receptor subtype, an important characteristic may be that brain IRs do not down-regulate in response to excess insulin (Boyd and Raizada, 1983; Zahniser et al., 1984). Taken together, differential posttranslational processing and lack of down-regulation for the brain IR suggests that the brain IR may have functions that are different from those of its peripheral counterpart.

The Canonical IR Signaling Pathway(s). Upon binding of insulin at the α subunit, the β subunit undergoes autophosphorylation, triggering a series of intracellular

phosphorylation events that can ultimately affect gene expression. Mediators of IR signaling are insulin receptor substrates (IRS), SH2 domain-containing proteins which in turn phosphorylate various downstream effector molecules such as the membrane-embedded regulatory subunit of phosphatidyl inositol 3 (PI3) kinase. PI3 kinase can activate PKC as well as PKB/Akt. Both have been implicated in a myriad of cellular processes including cellular energy homeostasis and survival, in part by phosphorylating and inactivating GSK-3β and controlling the balance of the Bcl family of proteins in a cell. Another pathway involves the mediator protein Shc, which links the IR to Grb-2/SOS complex without involvement of the IRS molecules. SOS is a GTP nucleotide exchange factor for Ras, which is a trigger kinase that activates the MAPK signaling cascade. The intracellular pathways are inter-dependent and can influence one another (e.g. Ras can stimulate PI3 kinase). It is often believed that the various actions of insulin are mediated through its glucoregulatory effects. The next section, however, will review the literature showing that insulin may have direct effects independent of glucoregulation.

## 1.8. IN VITRO STUDIES DEMONSTRATING NON-GLUCOREGULATORY ROLES OF INSULIN

*In vitro* Studies with Insulin on Neurons. Some of pioneering studies on selective effects of insulin were conducted on SH-SY5Y cells, a neuroblastoma cell line capable of surviving in serum-free medium for several days. Insulin concentrations as low as 0.1 nM were shown to be half-maximally effective at promoting neuritic sprouting, while anti-insulin antiserum inhibited that response (Recio-Pinto and Ishii, 1984). Further studies revealed that insulin promoted neurite outgrowth in part by increasing levels of α and β tubulin transcripts by selectively stabilizing these mRNAs rather than increasing the rate

of transcription (Fernyhough et al., 1989; Mill et al., 1985). Similarly, insulin concentration as low as 10 pM was effective at promoting neurite formation and survival of chick sympathetic neurons (Recio-Pinto et al., 1986). In agreement with the Ishii lab findings, others found that stimulation of protein synthesis in fetal chick neurons was found to be half-maximal with only 70 pM insulin, concentration far too low to cross-occupy the IGF-I receptor (Heidenreich and Toledo, 1989). Low concentrations of insulin were also reported to prevent serum deprivation-induced apoptosis in a neuronal cell line derived from the rat retina (Barber et al., 2001) and mouse cortex (Ryu et al., 1999) in a dose-dependent manner and through the PI3 kinase pathway.

*In vitro* Studies with Insulin on Glial Cells. In a cell culture comprised of mostly astrocytes, 7 nM insulin significantly stimulated protein synthesis in a dose-dependent manner, while maximal effect (145% above baseline) was reached with 18 nM (Clarke et al., 1985). By contrast, only 2 nM insulin was required to significantly increase transcription. In myelinogenic cultures of cells dissociated from embryonic mouse brain, low physiological concentration of insulin was found to increase the activity of myelin-building enzyme cerebroside sulfotransferase, as determined by measurements of incorporation of $^{35}S$ into sulfatide, a component of myelin (Ferret-Sena et al., 1990). Low physiological concentrations of insulin can also prevent serum withdrawal-induced apoptosis in cultured optic nerve oligodendrocytes (Barres et al., 1993). Addition of other trophic factors such as IGFs potentiates the survival effect.

Taken together, these results demonstrate that insulin selectively exerts trophic effects on both neurons and glia when present at low physiological concentrations. However, since the *in vitro* studies of insulin are generally conducted with embryonic cells in a milieu far removed from the brain, these studies reveal potential rather than actual physiological actions of insulin. The neurobiology of insulin can only be established by careful *in vivo* studies.

## 1.9. IN VIVO STUDIES ON POTENTIAL NON-GLUCOREGULATORY ROLES OF INSULIN IN MAMMALIAN BRAIN

Clinical Studies. Insulin has been implicated in cognitive processes in humans. Intravenous administration of insulin acutely raises insulin concentration in the brain and infusion of low doses of insulin under glycemic clamp in normal adults results in facilitation of memory (Craft et al., 2003). However, even though the glucose clamp keeps circulating blood glucose constant, it but does not reveal whether glucose utilization in brain is changing during treatments. This is a problem, because glucose administration alone can alter learning and memory. Administration of insulin intranasally, however, does not alter glycemic levels. Intranasal insulin administration over a period of 8 weeks was also found to improve declarative memory (immediate and delayed recall of word lists), attention (Stroop test), and mood in 38 healthy test participants where blood glucose and plasma insulin levels were not affected (Benedict et al., 2004). However, very high insulin doses are necessary to obtain the observed effects on learning and memory by intranasal administration, and it is unclear whether the improvements in memory are due to insulin cross-occupancy of the IGF-I receptors, stimulation of IGF production or other causes. Because a brain neuron-specific conditional knockout of the IR demonstrates no effect on learning and memory (Schubert et al., 2004), the observed effect may indeed be IGF-I receptor-mediated. Perhaps the largest impediment to the hypothesis that insulin directly regulates cognitive processes is that learning and memory would then be dependent on fluctuation in insulin levels following meals, which seems unlikely.

Experimental Studies. Insulin administration can also lead to enhanced learning and memory in animals. Healthy rats injected i.c.v. with 4 mU insulin following training in a passive-avoidance test chamber exhibited greater latency to enter the chamber where

they received a shock compared to rats which received saline vehicle or heat-deactivated insulin (Park et al., 2000). While the evidence implicating insulin in mediation of memory is compelling in this study, there is no proof that the observed effect is glucose-independent nor that insulin was exerting its effect through the IGF-I receptor rather than its own receptor.

Further support of insulin involvement in learning and memory comes from studies showing up-regulation of IR mRNA and IR protein content in synaptic membrane extracts from healthy rat hippocampi following spatial maze learning task (Zhao et al., 1999). The increased accumulation of IR protein in the hippocampal crude synaptic membrane fraction relative to cytosolic fraction was interpreted as receptor translocation to the membrane. IR tyrosine phosphorylation was increased in trained animals compared to controls, which was associated with co-immunoprecipitation of the IR with its downstream targets Shc and Grb.

In the STZ-diabetic rodent model of diabetes where insulin levels are reduced and learning and memory is impaired, subcutaneous treatment with insulin reverses learning and memory deficits in a T-maze (Flood et al., 1990), and Morris Water Maze (Biessels et al., 1998). Impairments in hippocampus-dependent long-term potentiation are also prevented with insulin treatment at the onset of diabetes (Biessels et al., 1998). Investigation of the NMDA receptor complex which mediates long-term potentiation reveals that there is a decrease in the relative abundance of NR2A and NR2B subunits that make up the receptor in the hippocampus of STZ rats (Delibas et al., 2004; Di Luca et al., 1999). Phosphorylation of the NR2A subunit of the receptor complex is reduced concomitantly with impairments in NMDA receptor-mediated currents (Gardoni et al., 2002). In all cases, subcutaneous insulin administration prevented these changes. However, since hyperglycemia was lowered in all these studies, normalization of the

measured parameters by insulin treatment cannot exclude hyperglycemia as the main pathogenic factor.

Insulin may exert its effects on learning and memory by modulating brain neurotransmitter levels. There is a marked increase in acetylcholine and serotonin and a concomitant decrease in dopamine and noradrenaline following i.c.v. injection of insulin into rat brain (Bhattacharya and Saraswati, 1991). *In vivo* studies provide evidence that insulin may regulate synthesis and clearance of these neurotransmitters by regulating transporters for dopamine, serotonin, and GABA (Figlewicz, 1999).

In experimental brain injury, subcutaneous administration of insulin in rats subjected to brain ischemia resulted in significant reduction in the number of necrotic cells (Voll and Auer, 1991). These effects were independent of hypoglycemia because protection was observed even when glycemic levels were maintained by a glucose clamp. Diabetes is well known to exacerbate ischemic damage and in STZ-diabetic rats subjected to ischemia, chronic subcutaneous insulin treatment decreased the basal apoptotic level (Rizk et al., 2006). Both acute and chronic insulin decreased the ischemia-induced lesion volume and apoptosis. However, subcutaneous insulin also in this case reduced the glycemic levels and the high dose of insulin used in this study may implicate the IGF receptors.

In summary, while there are clinical and experimental reports implicating insulin in learning and memory as well as experimental data demonstrating a role of insulin as a trophic factor, no evidence is provided that the observed effects are hyperglycemia- and IGF receptor-independent.

## 1.10. EVIDENCE AGAINST THE INSULIN RECEPTOR AS BRAIN NEURONAL GLUCOREGULATOR

Because brain energy utilization is closely tied to function, one may not desire direct regulation by insulin that fluctuates in concentration with meals. Several lines of evidence suggest that insulin receptors do not regulate glucose utilization in brain. While binding of insulin to IR in muscle or adipose tissue stimulates translocation of specialized transporter proteins (GLUTs, most prominently GLUT4) to facilitate influx of glucose, brain neurons appear to possess other mechanisms by which they obtain glucose, including transporters that are insulin-independent (Schulingkamp et al., 2000).

Clinical Studies. Magnetic resonance imaging (MRI) examination of human brain found that intravenous infusion of insulin under glycemic clamp did not alter glucose utilization in gray matter-rich occipital cortex and white matter-rich periventricular brain tissue (Seaquist et al., 2001). In addition, positron emission tomography (PET) scans of brain glucose utilization in type I diabetic patients showed that glucose utilization was not dependent on physiological concentrations of administered insulin (Cranston et al., 1998).

Experimental Studies. In agreement with clinical findings, intravenous infusion of insulin into rats under steady-state normoglycemic conditions was ineffective in altering glucose utilization in brain (Lucignani et al., 1987). An exception, however, were the ventromedial, dorsomedial, and anterior hypothalamic nuclei that are known to control feeding and satiety (Schwartz et al., 1992). In *in vitro* studies involving chick brain neurons where low insulin concentration induced neurite outgrowth, neither glucose transport, glucose oxidation, nor glycogen synthase activity were affected (Heidenreich et al., 1988). On the other hand, low insulin concentrations were found to promote glucose uptake in glial cells cultured from neonatal rats (Clarke et al., 1984). The

observed differences in negative cooperativity and posttranslational modification between the neuronal and glial IR subtype discussed earlier may account for these cell type-specific differences in function.

$^{14}$C-deoxyglucose autoradiography and *in situ* hybridization studies on postnatal brain slices demonstrated that the neuroanatomical pattern of glucose utilization does not reflect IR gene expression (Bondy and Cheng, 2004). Interestingly, there was a strong overlap between glucose utilization and IGF-I receptor expression in these brains, suggesting that IGFs may promote glucose utilization at certain stages of development. Indeed, the possibility of IGFs acting in a compensatory fashion to insulin was suggested following findings in the NIRKO mouse, where the selective knockout of the neuronal IR in brain did not account for any change in glucose utilization, brain degeneration, nor learning and memory impairments.

Taken together, insulin is not responsible for the bulk of glucose utilization in the brain, suggesting an alternate role for the widespread distribution of the IR. There are indications that insulin may affect learning and memory as well as cell survival, although secondary effects of insulin such as on glycemic levels and likely cross-occupancy of the IGF-I receptor can not be ruled out as contributing factors. There is a possible redundancy provided by the IGF system, since the NIRKO mouse shows no abnormalities in neuronal survival and learning and memory (Bruning et al., 2000; Schubert et al., 2004). The roles of IGFs in the brain are introduced next.

## 1.11. BRAIN INSULIN-LIKE GROWTH FACTORS (IGFs) ARE REDUCED IN DIABETES

Sources of Cerebral IGFs. IGF-I and IGF-II are members of a highly conserved insulin gene family that structurally resemble pro-insulin (Russo et al., 2005). Their activity is highly regulated due to binding to six different IGF binding proteins (IGFBPs). About 1% of plasma IGF-I circulate in the free form, while the remainder are bound to IGFBPs that sequester and prolong the half-life of the IGFs (Janssen and Lamberts, 1999; Jones and Clemmons, 1995). Specific proteases are required to cleave the IGF peptide from its binding protein in order for the IGFs to bind to receptors and exert biological effects.

IGFs cross the blood-brain-barrier and the uptake is saturable, indicating receptor-mediated transport (Armstrong et al., 2000). The uptake appears to be IGF-I receptor-independent, since IGF-I analogs with multiple times lower affinity for the IGF-I receptor competed for the uptake (Pulford and Ishii, 2001). Instead, a multicargo endocytic receptor called megalin is believed to mediate the uptake of both insulin and IGF-I at choroid plexus into brain (Carro et al., 2005).

IGFs are also expressed in the brain itself. The most abundant IGF in the brain is IGF-II, which is synthesized in discrete brain regions such as the leptomeninges and the choroid plexus (Bondy and Lee, 1993). By contrast, most brain IGF-I is believed to originate in the liver (Murphy et al., 1987), even though there is evidence of local IGF-I expression in the brain, particularly in the olfactory bulb, hippocampus, and cerebellum (Werther et al., 1990). While during development IGF-I gene expression appears to be exclusively neuronal, astrocytes tend to express both IGFs in response to injury in adult brain (Bondy and Cheng, 2004). IGF-I mRNA levels in rodent brain are relatively modest prenatally, but increase during early postnatal period and decline again after a

few weeks (Bondy, 1991). In addition to IGF-I, the brain expresses an alternate splice variant called des(1-3)-IGF-I, a brain homolog of IGF-I which exhibits higher potency, probably due to its inability to be bound and sequestered by the IGFBPs (Sara et al., 1993).

IGF Levels are Reduced in Clinical Diabetes. Circulating IGF levels slowly decline over decades in normal (Hall and Sara, 1984) and diabetic subjects (Tan and Baxter, 1986) and are reduced approximately 50% at any age in type I and type II diabetic patients compared to age-matched non-diabetic subjects. The decline in circulating IGF-I levels was reported to be even greater in diabetic patients with progressive neuropathy and IGF availability is diminished further due to altered binding to IGF binding proteins (Arner et al., 1989; Migdalis et al., 1995). Depending on duration and severity of diabetes, circulating IGF-II levels can also be reduced (Amiel et al., 1984; Bowsher et al., 1991). These data show that IGF levels are lower in diabetic than normal subjects, and that such levels continue to decline with aging. This may be related to the increased risk for cognitive disturbances with aging.

IGF Levels are Reduced in Experimental Diabetes. Similarly to the clinical situation, IGF levels are reduced in STZ rodents. Liver is the main source of circulating IGFs and hepatic IGF-I mRNA content decreases rapidly over a period of 3 days after STZ administration (Fagin et al., 1989; Yang et al., 1990). Even though IGF-II is the predominant IGF in the brain, rodents do not express IGF-II in liver due to absence of the hepatic promoter. IGF-II is expressed in discrete brain regions such as leptomeninges and choroid plexus. Levels of IGF-II mRNA are reduced in brain and spinal cord after 2 weeks of STZ-diabetic rats as well as in leptin receptor-deficient *fa/fa* obese Zucker rats, a genetic model of type II diabetes (Wuarin et al., 1996). IGF and IGF-I receptor mRNA content in brain is reduced also in BB/Wor rats, a genetic model of type I diabetes (Li et al., 2002). Circulating IGF-I protein levels are reduced in STZ rats

by about half in some experiments (Busiguina et al., 2000; Pao et al., 1992) and more than 80% in others (Scheiwiller et al., 1986), resembling IGF levels in clinical diabetes. In addition, IGF-I levels are reduced in cerebellum and hypothalamus of STZ rats (Busiguina et al., 2000; Olchovsky et al., 1991). The reduction in IGF availability in STZ mice is associated with a down-regulation of both mRNA and protein levels of PI3 kinase and PKB/Akt, as well as perturbations in the intracellular signaling cascade (Francis et al., 2008).

These data show that there is a reduction in circulating and brain IGF levels in both clinical and experimental diabetes. The decline in IGF levels is greater with aging and with development of diabetes. The reduction in IGF levels may not be due to hyperglycemia, because hepatic cells respond directly to insulin in culture (Goya et al., 2001; Krishna et al., 1996; Phillips et al., 1991). In addition, peripheral administration of IGF-I can prevent the decline in IGF-II gene expression in brain in STZ diabetes independently of hyperglycemia (Wuarin et al., 1996).

## 1.12. BRAIN IGF RECEPTORS

The IGF-I receptor is structurally closely related to the IR. While both IR and IGF-I receptor are $\alpha\alpha\beta\beta$ tyrosine kinases, the IGF-II receptor is structurally unrelated to that receptor family and does not exhibit tyrosine kinase activity. Also known as the mannose-6-phosphate receptor, the IGF-II receptor binds exclusively IGF-II with high affinity and its sole function appears to be in regulation of IGF-II availability. Biological effects of both IGF-I and IGF-II are believed to be mediated through the IGF-I receptor, since the IGF-I receptor binds also IGF-II in addition to IGF-I, albeit with a 2-10-fold lower affinity (Schulingkamp et al., 2000). IGF-I receptor mRNA is very high in brain

over the course of maturation, but decreases during postnatal development as a percent of total brain RNA (Werner et al., 1989). Expression remains relatively high, however, in the choroid plexus and leptomeninges. As cells differentiate, IGF-I receptors concentrate more on neurons as opposed to glia (Bondy and Lee, 1993). Relatively high concentrations of $^{125}$I-IGF-I receptor binding sites are observed in the olfactory bulb, cortex, hippocampus, choroid plexus, median eminence, and in the granular as well as molecular layers of the cerebellum of adult rodents. These findings are largely in agreement with temporal and spatial IGF-I receptor mRNA distribution (Kar et al., 1993). Electron microscopy investigation of the IGF-I receptor in adult rat cerebellar cortex and hypothalamic arcuate nucleus shows that the receptors are found on both neurons and glia and that their sub-cellular distribution includes pre- and post-synaptic regions (Garcia-Segura et al., 1997). Much like the IR, IGF-I receptor in the brain is somewhat smaller than its peripheral counterpart. While the size of the glial IGF-I receptor largely resembles the peripheral IGF-I receptor, neuronal IGF-I receptors are smaller (Burgess et al., 1987).

IGF-Mediated Signaling Pathways. Intracellular pathways that are triggered by the IGF-I receptor are indeed insulin-like, activating virtually the same downstream effector molecules following ligand binding and receptor autophosphorylation (see previously introduced IR signaling, p. 17). In a yeast two hybrid assay that screened for proteins that selectively interact with the IGF-I receptor but not IR, only the adaptor protein Grb10 and two isoforms of protein 14-3-3 were identified (Lopaczynski, 1999). It remains unclear which physiological or biochemical consequences that selective activation may have. Given the promiscuity of these two proteins in their interactions with other cellular constituents, there may indeed be no fundamental difference in intracellular signaling pathways between the two receptors. For a more in-depth review of the signaling pathway mediated by the IGF-I receptor, the reader is referred to a

review (Bondy and Cheng, 2004). The common intracellular signaling pathway shared by IR and IGF-I receptor supports the possibility that concomitant decline of both ligands is needed to precipitate brain atrophy.

## 1.13. ROLES OF IGFs IN THE BRAIN

Most of the present understanding of the neurobiology of IGFs in brain is derived predominantly from developmental studies. Much less is known about the normal role of IGFs in the adult brain.

IGFs in Clinical Studies. In human brain development, IGF-I was found to be significantly elevated in young children cerebrospinal fluid (<6 months) compared to older children, while IGF-II is higher in older children. This is in contrast to peripheral circulation where IGF-I is low at birth, rises rapidly during the 1$^{st}$ year, and reaches a peak at puberty (Bunn et al., 2005). Circulating IGF levels decrease slowly thereafter as aging progresses (Hall and Sara, 1984). Human cognitive performance correlates with serum IGF-I levels and age (Rollero et al., 1998), which may explain why *Igf -/-* individuals exhibit severe mental retardation (Woods and Savage, 1996). Neuropsychological tests of visuoconstructive ability, perceptual-motor speed, mental tracking, and verbal long-term memory are known to decline with aging and lower IGF levels are reported to correlate with perceptual-motor and mental processing speed in the elderly (Aleman et al., 1999). A study that investigated visual and auditory learning in 56 healthy male subjects ages 21-84 found that the reduction in the ratio of circulating IGF-I to growth hormone (IGF-I/GH) correlated with age and learning impairments (Morley et al., 1997). The impact of reduced circulating IGF levels with aging are perhaps best demonstrated in prospective population-based studies where the same

subjects are investigated as time progresses. In one such study, higher serum IGFBP-bound IGF-I levels and higher total IGF-I/IGFBP-3 ratios were associated with less cognitive decline over a period of 2 years in a sample population of 168 healthy participants 55-80 years of age (Kalmijn et al., 2000). In a similar 3-year longitudinal study which involved a larger sample pool (1318 subjects, 65-88 years of age), low serum IGF-I levels were found to be associated with deficits in information processing speed (Dik et al., 2003).

These studies show that there is an age-dependent decrease in circulating IGF levels in humans and that such decrease is associated with cognitive decline. On the other hand, there are very few IGF intervention studies performed on humans.

IGF in vitro Studies. A plethora of studies have investigated pleiotropic roles of IGFs *in vitro* (Russo et al., 2005). IGFs were found to promote mitogenesis of astrocytes (Tranque et al., 1992), proliferation of microglia (O'Donnell et al., 2002), oligodendrocyte differentiation (Broughton et al., 2007), regulate dendritic arborization and neuritic sprouting of neurons (Niblock et al., 2000), and protect against cell death (Butts et al., 2003; Willaime-Morawek et al., 2005). While it is clear that IGFs exert pleiotropic effects on virtually all cell types, the *in vitro* environment is far removed from the normal biochemical milieu of the brain and only inferences can be made with respect to IGFs' true biological roles *in vivo*.

Brain IGFs in Experimental Studies in Development. More comprehensive understanding of the roles of IGFs in developing mammalian brain have been acquired with genetic mouse models that either over-express or lack IGF or IGF-I receptor gene expression. Transgenic mice ectopically over-expressing the IGF-I gene during later development under a metallothionein promoter reveals significant increases in brain size and myelin content, resulting in brain mass increased by 50% (Carson et al., 1993; D'Ercole, 1993; Ye et al., 1995). The increase in brain mass is largely attributed to

enlarged somas, axonal diameters and increased total myelin content, as well as a diminished incidence of apoptosis during the developmental pruning period of superfluous cells (Chrysis et al., 2001). Interestingly, the increase in neuropil was largely attributed to increased myelin production per oligodendrocyte rather than increased oligodendrocyte proliferation (Carson et al., 1993).

Injection of IGF-I into rat cerebrospinal fluid twice daily from P6 to P9 had no effect on the number of promyelinating oligodendrocytes, but essentially doubled the number of myelinating oligodendrocytes and myelin sheaths (Goddard et al., 1999). That was associated with a concomitant increase in CNPase, an enzyme implicated in myelin biosynthesis. Thus, experiments where endogenous IGF expression is increased or where exogenous IGFs are added show that IGFs increase brain mass primarily by stimulating myelin synthesis but also by preventing programmed cell death.

IGF-I knockout mice demonstrate essentially the opposite effect. Targeted deletion of the brain *Igf1* gene results in reduced brain mass evenly affecting all major brain areas. At P40, time at which myelination is considered complete, brain mass is reduced by a about one third compared to wild-type littermates. There is a roughly proportionate decrease in brain protein and myelin contents (Cheng et al., 1998). Soma size, dendritic length and arborization of projection neurons is reduced. Hypomyelination is evident due to decreased size of myelin tracts. Interestingly, there is a proportionate decrease in the number of oligodendrocytes to reduced number of axons and medium neurofilament abundance (Beck et al., 1995; Cheng et al., 1998; Ye et al., 2002). Myelin composition with respect to MAG and MBP is not significantly different in the knockout mice compared to controls when normalized to brain weight or total brain protein content, although their expression is reduced (Ye et al., 2002). In addition, certain cell populations are reported to be lower in number, possibly as a consequence of diminished differentiation, proliferation and/or survival. Homozygous IGF-I null mice

are born with less than 60% body weight and most die before reaching adulthood (Powell-Braxton et al., 1993). It is interesting to note that glucose utilization is significantly reduced in the *Igf1-/-* brain, particularly in structures where *Igf1* expression is normally most abundant (Cheng et al., 2000), suggesting a glucoregulatory role for IGFs. Mice in which the IGF-I receptor gene (*Igf1r*) is conditionally ablated either in Olig1- (developmental marker of oligodendrocytes) or proteolipid protein (component of myelin)-expressing cells demonstrate fewer oligodendrocyte precursors after 2 weeks (during myelination), up to one quarter fewer mature oligodendrocytes after 6 weeks (myelination mostly complete) and 25 weeks (adulthood) as a result of decreased proliferation and increased apoptosis (Zeger et al., 2007).

Double knockout of genes encoding for both IGF-I and IGF-II is invariably lethal post birth and knockout of the IGF-I receptor resembles that phenotype (D'Ercole et al., 2002; Liu et al., 1993). That is expected, because both IGFs are believed to mediate their biological effects through the IGF-I receptor.

In summary, these reports demonstrate that IGF activity is important *in vivo* in cellular proliferation and survival during development, particularly with regard to oligodendrocytes and myelination. IGFs are a regulator of brain mass in development.

Brain IGFs in Experimental Studies with Adult Animals. Disruption of IGF activity in the adult rat brain has been found to adversely affect learning and memory. Injection of IGF-II antiserum into lateral brain ventricle of adult healthy rats results in learning and memory impairments in a passive-avoidance test, while injection of pre-immune serum has no effect (Lupien et al., 2003).

Dendritic spines form synapses which believed to be the cornerstone of learning and memory. Injection of IGF-I antisense RNA in the inferior olive of adult rats elicits a significant and reversible decrease of IGF-I levels in the contralateral cerebellum. Electron microsopy ultrastructural analysis of the cerebellar cortex showed a significant

reduction in the size of dendritic spines on Purkinje cells of antisense-treated rats compared to controls. The decrease in spine size was accompanied by a decreased density of spines on Purkinje cells (Nieto-Bona et al., 1997). This study mechanistically implicates IGFs in modulation of learning and memory through spine plasticity in the adult rat.

Injection of a cDNA viral vector encoding a dominant-negative IGF-I receptor into adult rat brain lateral ventricle results in dimerization of the kinase-dead subunit with the endogenous IGF-I receptor subunits, which disrupts the endogenous choroid plexus IGF-I receptor activity. The outcomes are cognitive impairments associated with synaptic vesicle protein loss, as well as neuropathological changes reminiscent of Alzheimer's Disease (Carro et al., 2006). Disruption of the choroid plexus IGF-I receptor in LID mice (liver-specific IGF-I knockout animals that results in 60% reduction in circulating IGF-I) results in similar pathological changes in the brain (Carro et al., 2006).

IGFs improve learning and memory in aged rats. Rats 4 and 32 months of age were preoperatively trained in behavioral tasks and subsequently implanted with osmotic minipumps to i.c.v. infuse IGF-I or vehicle. Animals were retested at two weeks and four weeks after surgery. Animals injected with IGF-I exhibited improved working memory in the repeated acquisition task, object recognition task, and place discrimination task compared to vehicle-receiving controls (Markowska et al., 1998). In a separate study, i.c.v. IGF-I infusion in aged rats over a period of 3 weeks resulted in prevention of age-dependent decrease of NR2A and NR2B NMDA receptor subunit expression (Sonntag et al., 2000). This finding is important, because a disruption of the NR2B subunit expression with antisense oligonucleotides diminishes NMDA receptor-mediated long-term potentiation (LTP) and impairs spatial learning (Clayton et al., 2002).

IGFs are known to prevent apoptosis and promote regeneration in the adult rodent brain. In rats subjected to unilateral hypoperfusion injury, i.c.v. administration of 5

to 50 μg IGF-I two hours after injury was shown to be neuroprotective in a dose-dependent manner in all regions (Guan et al., 1993), whereas pretreatment with IGF-I did not alter the outcome. These determinations were made through histological examination of slices from the cortex, striatum, and hippocampus 5 days after injury and treatments. The study also shows that IGF-I appears to be a more potent neurotrophic agent than insulin, since IGF-I was more completely neuroprotective compared with equimolar doses of insulin. In these models of hypoxic-ischemic brain injury, i.c.v. $^{3}$H-IGF-I is rapidly translocated to neurons and glia in injured sites, a signal that persists for up to 6 hours after administration, suggesting local storage of the growth factor (Guan et al., 2000). Interestingly, neither IGF-II nor des(1-3)IGF-I was neuroprotective (Guan et al., 1996), which the authors speculate may be attributed to their inability to bind IGF binding proteins that normally protect IGF-I from degradation. IGFs have also been implicated in re-myelination following injury in adult mice. Cuprizone-induced apoptosis of mature oligodendrocytes in adult mice is followed by oligodendrocyte progenitor proliferation and migration to lesioned sites, a process which is accompanied with a strong increase in IGF-I mRNA expression in the brain (Mason et al., 2000).

In addition to increasing cellular proliferation in models of brain injury, IGFs have been implicated in stimulating proliferation in hypophysectomized adult rodents. Hypophysectomized rodents lack the pituitary and as a consequence have severely reduced IGF production due to lack of growth hormone. Peripheral administration of IGF-I into adult hypophysectomized rats stimulates neurogenesis in the granule cell layer of the hippocampus, brain region where some neurogenesis persists into adulthood. The number of BrdU-incorporating neurons was increased by 78% (Aberg et al., 2000). IGFs stimulate survival of these newborn cells, as well as an increase in oligodendrogenesis in the cortex (Aberg et al., 2007). Interestingly, the increase in the number of new neurons in the hippocampus in adult rats can also be stimulated by

exercise, and blocking peripheral IGF-I uptake into the brain completely inhibits exercise-induced increase in the number of newborn neurons (Trejo et al., 2001).

As in development, IGFs have been implicated in glucose utilization in adulthood as well. Brain glucose utilization is significantly decreased in aged rats throughout the brain, particularly in cortex, hippocampus, and several regions of the hypothalamus. Infusion of 100 ng/μl IGF-I i.c.v. for a period of 4 weeks increases utilization of $^{14}C$-deoxyglucose in these brain regions compared to saline-receiving age-matched group (Lynch et al., 2001). This suggests that IGFs may modulate glucose utilization in adult brain, although the authors do not rule out the possibility that the effects could be secondary to other processes rather than direct IGF action.

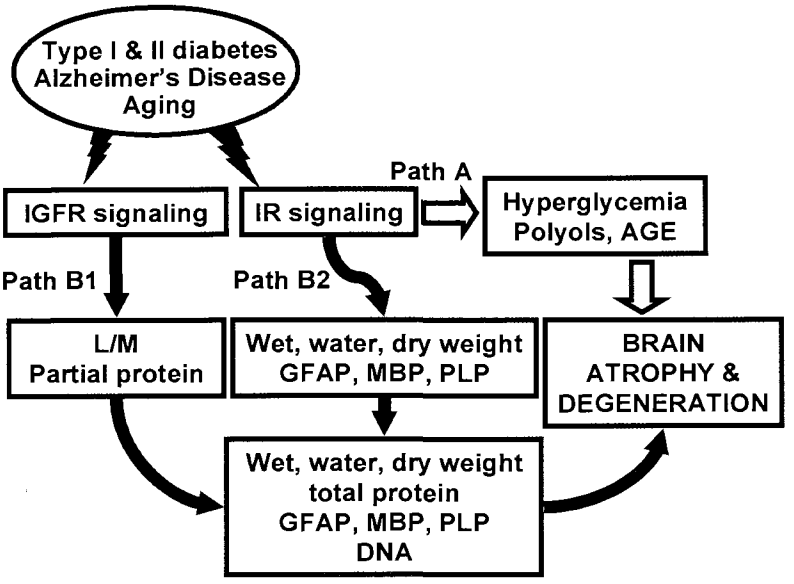
Taken together, IGFs have been demonstrated to protect from cell death, stimulate proliferation, and modulate learning and memory in adult mammalian brain.

## STATEMENT OF HYPOTHESIS

### STATEMENT OF ALTERNATIVE HYPOTHESES FOR PATHOGENESIS OF BRAIN ATROPHY IN DIABETES

The Introduction has provided a review of the literature showing that insulin and IGFs have neurotrophic activites, and the concomitant decrease in insulin and IGF signaling may contribute at least in part to the pathogenesis of diabetic brain abnormalities. Insulin at normal physiological concentrations does not appear to regulate glucose utilization in brain, and the role of the widespread insulin receptors in brain remains unexplained. Knockout of the neuronal insulin receptor (the NIRKO mouse) has no effect on glucose utilization, learning and memory nor is there evidence of brain degeneration. We propose that IGF may compensate for the loss of insulin activity in such knockout mice, and a central feature of our hypothesis is that increased risk for brain atrophy involves the concomitant decline in insulin and IGF activities. The loss of insulin activity may contribute to atrophy as a consequence of its reduced growth factor rather than glucoregulatory activity.

Diabetes is a disease where there is brain atrophy and degeneration in association with electrophysiological and learning/memory impairments. The STZ rat is a reasonable model that displays similar pathology. In addition, the STZ-diabetic rodent model allows for testing of the below proposed alternative hypothesis because there is concomitant reduction in insulin and IGF activity. This hypothesis is independent of type of diabetes because biological activity of insulin and IGFs are diminished in both type I and type II diabetes. Since impairments in insulin and IGF activity is a hallmark of clinical late onset Alzheimer's disease (see Discussion, p. 124), this study may have implications for Alzheimer's disease as well.




**Hypothesis figure.** Alternative hypothesis for the pathogenesis of brain atrophy in a model of concomitantly reduced insulin and IGF brain activity.

The alternative hypothesis for pathogenesis of brain atrophy in physiological conditions of concomitantly reduced insulin and IGF activity is presented in Fig. 2.1.

We hypothesize that:

1) tiny replacement doses of insulin, IGF-I, or a combination of insulin and IGF-I administered intracerebroventricularly (i.c.v.) in adult STZ rats can prevent brain atrophy and degeneration (Paths B1 and B2),
2) insulin and IGF-I act in synergy to more completely prevent brain atrophy and degeneration than either factor alone,
3) insulin and IGF-I prevent brain diabetes-associated abnormalities independently of ongoing hyperglycemia (Path A). We will test these hypotheses by keeping hyperglycemia constant while stimulating the growth factor pathway.

## PART 2. MATERIALS AND METHODS

### 2.1. DIABETIC RAT TREATMENT

The NIH Guide for the Care and Use of Laboratory Animals was followed, and the animal protocol was approved by the Animal Care and Use Committee of Colorado State University. Wistar rats (275-300 g adult 9 week old males, Harlan Laboratories) were randomly assigned to treatment groups. Untreated rats were in the control group (Non-D, N=8 rats), whereas 50 mg STZ per kg body weight was used to induce type I diabetes in other rats as previously described (Lupien et al., 2003). STZ-treated rats with >20 mmol/L (360 mg/dL) glucose were enrolled in the study. Food and water were available *ad libitum*. Recombinant human IGF-I (Gropep, Australia) and human insulin (Sigma) were purchased. During the induction of diabetes, rats were deeply anesthetized with 110 mg/kg ketamine and 8 mg/kg xylazine i.p. and implanted subcutaneously in the mid-back with osmotic minipumps (Durect Corp., Cupertino, CA) that continuously released either vehicle or treatment at a rate of 12 µl/day.

In the "12 week insulin" experiment, pumps were delivering either 510 pmol/day insulin (D+ins, N=9), the combination of 510 pmol/day insulin plus 65 pmol/day of IGF-I (D+Ins+IGF, N=9) or vehicle artificial cerebrospinal fluid (D+aCSF, N=9) comprised of 10 mM NaCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM $K_2HPO_4$, and 10 mM glucose, pH 7.4 for 12 weeks. The number of animals in the control non-diabetic (Non-D) group was 8.

The "12 week IGF" experiment was performed several months after the "12 week insulin" experiment. Pumps were delivering the same solutions, except that the insulin-alone pump was replaced by the IGF-alone pump administering the same IGF concentration as the combination (65 pmol/day). Group numbers for the IGF experiment were as follows: ND, N=9; D+aCSF, N=9; D+IGF, N=10; and D+ins+IGF; N=9. In both experiments the osmotic pumps were connected to a subcutaneous catheter that was

routed to a cannula implanted into the left brain lateral ventricle (coordinates: -0.9 mm caudal and 1.5 mm left from bregma; 5 mm deep) using an Alzet Brain Infusion Kit (Durect Corp., Cupertino, CA). A rat stereotaxic instrument with micromanipulator and brain atlas were used (Paxinos and Watson, 1986). Buprenorphine 30 µg/kg was used beginning 1 day prior to and 3 days after surgery to provide postoperative analgesic care. At two week intervals, tail blood was drawn, and minipumps and solutions were replaced under brief isofluorane anesthesia. Correct placement in the ventricle was confirmed by injecting 20 µl dye through the catheter at time of assay. At the conclusion of the experiment, rats were weighed and deeply anesthetized with 110 mg/kg ketamine and 8 mg/kg xylazine. CSF was withdrawn at the cisterna magna for glucose and ELISA assays, then rats were euthanized with excess carbon dioxide while anesthetized. Kidney wet weights were measured. The brain (except olfactory bulb) was separated from the spinal cord with a single perpendicular knife cut at the caudal edge of the cerebellum, and wet weights of brain were taken. Each brain was split down the midline and each brain half was weighed, frozen in liquid nitrogen, and stored at -70°C. Brain halves were homogenized with a Tissue Tearer (Fisher) in 10 ml buffer containing protease inhibitors: leupeptin, chymostatin, pepstatin A (10 µg/ml each), and 0.1 mM phenylmethylsulfonylfluoride in 10 mM tris acetate and 5 mM EDTA, pH 7.4. Homogenates and all tissues were stored in cryotubes at -70°C.

## 2.2. DRY AND WATER WEIGHTS

Aliquots of tissue homogenates, taken after trituration 30 times, were lyophilized to measure dry weight in duplicate. The weight of an equivalent volume of buffer alone was subtracted from sample weights. The water content was the difference between tissue wet and dry weights.

## 2.3. DNA ASSAY

Aliquots of brain tissue homogenates were thawed, triturated 30 times, and combined with Hoechst 33258 dye. Hoechst dye binds to the AT rich regions of double-stranded DNA and exhibits enhanced fluorescence under high ionic strength conditions. Sensitivity of the Hoechst 33258 Dye assay is approximately 10 ng/mL and the linear dynamic range extends over 3 orders of magnitude. Samples were measured in quadruplicate using VersaFluor Fluorometer (Bio-Rad) (excitation 360 nm; emission 460 nm). Calf thymus DNA was used for concentration standard curves. The strength of this method is that it provides a quantitative assessment of relative cell number *remaining* at the conclusion of the experiment (12 weeks). However, this method cannot reveal the manner by which cells are lost (apoptosis or necrosis) nor can it discriminate between cell types.

## 2.4. PROTEIN ASSAY

Aliquots of tissue homogenates (after trituration 30 times) were solubilized with >1.4 g SDS/g protein and each sample was applied onto a 1.5 $cm^2$ square on Whatman #4 filter paper grid. Paper sheets were allowed to dry, immersed in methanol to fix the proteins, dried, and briefly incubated in a Coomassie dye solution. Excess Coomassie was washed out and the filter paper was allowed to dry. Each square was cut and placed into a microfuge tubes to which the Assay Solution was added to extract the bound Coomassie (66% methanol, 1% ammonium hydroxide). Tubes were vortexed and allowed to incubate for 30 minutes. An aliquot of the dye solution was applied onto a 96-well plate and protein content was measured on a microplate reader by subtracting the absorbance at 405 nm from the absorbance at 600 nm (Minamide and Bamburg, 1990). Bovine serum albumin was used to construct the standard curve.

Prior to performing the total protein assay, an experiment was performed to determine the optimal solvent for the brain homogenate that would allow for maximum protein recovery in the assay. The following buffers were tested:

1) BHB; brain homogenization buffer, consisting of 10 mM tris acetate and 5 mM EDTA pH 7.4, 0.1% SDS, 0.1% triton X-100. This is the buffer that the rat brain tissue was homogenized in.
2) SDS; saturating sodium dodecyl sulfate concentration, 1.4 g SDS per gram estimated protein content dissolved in BHB.
3) GLB; gel loading buffer used to run Western blots: 50 mM Tris-HCl, 10% glycerol, 2% SDS, 4 M urea in BHB.

Eight independent samples were prepared for each group from the same homogenate and applied onto Whatman filter paper squares as described above. The results are shown in table 2.4.1. below. In addition, applied samples are shown on Whatman paper prior to dye extraction. Notice precipitated protein in BHB-extracted samples (red box), whereas no precipitate is visible in SDS and GLB-extracted homogenate samples. Given these results, saturating SDS concentration was determined to be optimal in extracting the maximal protein from brain homogenates.

**Table 2.4.1.:** test run determining optimal extraction solution for protein content determination in brain homogenates. Mean absorbance values ± S.E.M. and protein content per brain.

| Sample I.D. | Mean absorbance | **Prot./brain (mg)** |
|---|---|---|
| BHB | 0.388 ± 0.028 | **253.5** |
| SDS | 0.466 ± 0.020 | **343.4** |
| GLB | 0.434 ± 0.019 | **299.2** |

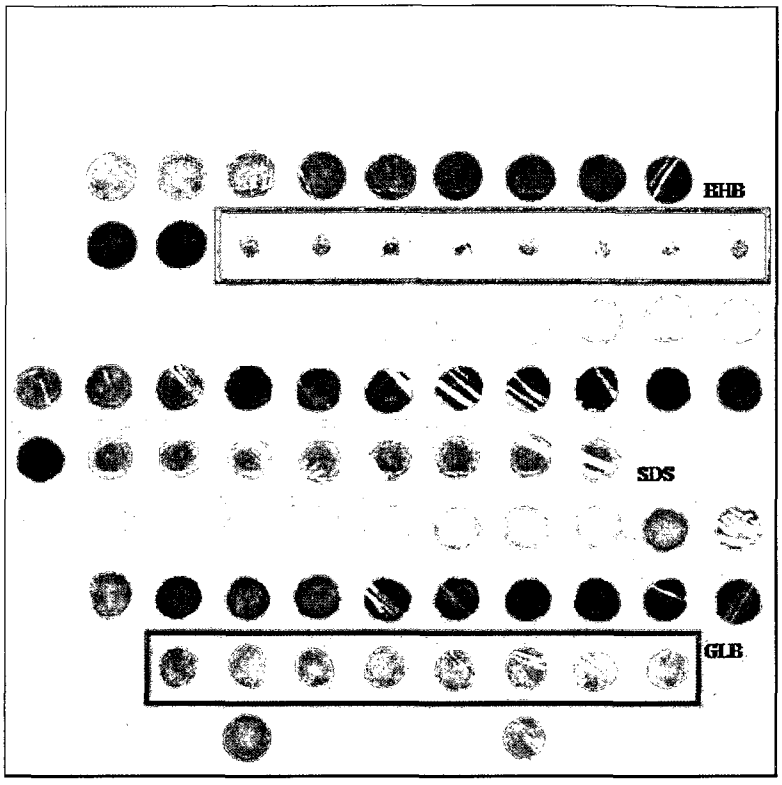



**Fig. 2.4.1.:** filter paper grid with applied BSA standards and brain homogenate samples where protein was extracted with either BHB, SDS, or GLB.

## 2.5. GLUCOSE ASSAYS

Plasma and CSF glucose concentrations were measured in duplicate using a glucose oxidase colorimetric assay (Sigma). Briefly, appropriately diluted plasma or CSF sample was allowed to react with glucose oxidase to produce hydrogen peroxide, which then oxidized o-dianisidine to produce a brown solution. Product's absorbance was measured on a spectrophotometer at 450 nm. A glucose standard solution was used to construct the standard curve.

## 2.6. WESTERN BLOTS

Certain standard Western blotting protocols may be inappropriate where there is brain atrophy. For example, the same amount of protein may be loaded per gel lane, but

this is questionable in diabetes where total protein levels are reduced. Levels of antigens on Western blots are often reported relative to total brain protein, actin levels, wet weights, or DNA. Yet the levels of all of these normalization parameters are changed in brain atrophy (see Results). Suppose that the left cortex, or every second brain cell, were completely lost but remaining brain cells were otherwise healthy. None of the forgoing standard protocols are likely to detect these losses. To avoid such concerns, present Western blot results were expressed on a per brain basis and gel lanes were loaded as equivalent fractions of whole brain homogenate.

Brain homogenates were thawed and triturated 30 times to assure a homogenous mixture. Aliquots were heated for 5 min at 40°C in gel loading buffer containing β-mercaptoethanol, 2% SDS and 4 M urea, and centrifuged at 10,000 x g for 30 min. Urea increased signal-to-noise ratios and reduced standard deviations. This treatment solubilized the samples so that neither precipitates nor cloudiness was visible prior to centrifugation. No pellet was visible after the spin, and aliquots of the supernatant solutions were loaded onto appropriate porosity polyacrylamide gels, depending on the size of antigen of interest.

To accommodate all samples (35 samples from "12 week insulin" experiment and 37 samples from "12 week IGF" experiment), four 15-lane gels were simultaneously run in a Mini-PROTEAN Tetra Cell (Bio-Rad), and three lanes of each gel contained a replicate non-diabetic normalization standard. The mean signal intensity of the three replicate standards was used to normalize values between the four blots. Following SDS-PAGE, samples were transferred to high binding capacity Immobilon-$P^{sq}$ PVDF membranes (Millipore) using a Trans-Blot SD Cell (Bio-Rad) semi-dry blotting apparatus and the following buffer compositions: Anode Buffer I (0.3 M Tris, 10% methanol, pH 10.4), Anode Buffer II (25 mM Tris, 10% methanol, pH 10.4), and Cathode Buffer (25mM

Tris, 10% methanol, 40 mM glycine, pH 9.4). Membranes were allowed to dry over night and were re-wetted in methanol followed by water prior to antibody incubations.

Reproducibility test using glutamine synthetase antibody is shown in fig. 2.6.1., showing that our approach gives reproducible results:

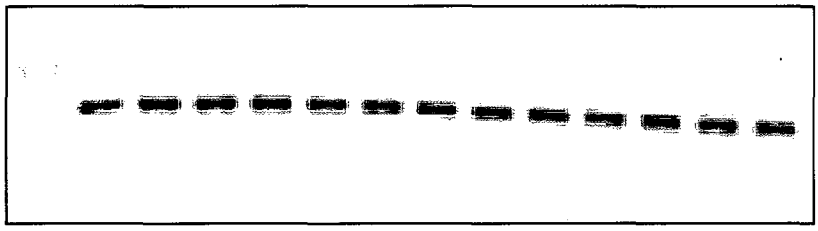


**Fig. 2.6.1.:** Western blot reproducibility test was conducted by applying replicate samples from a same stock brain homogenate prepared in parallel. Lane 1: molecular weight markers, upper band 50 kDa and lower band 37 kDa. Densitometric analysis of the bands revealed that the standard deviation was 10%, which can be largely attributed to pipetting error and perhaps variation in transfer to PVDF membrane.

Following manufacturer-recommended primary antibody dilution, a two-fold serial dilution of the SDS/urea supernatant from a Non-D rat was used to determine the linear and non-saturating range for each antigen investigated. An example using β tubulin antibody is provided:

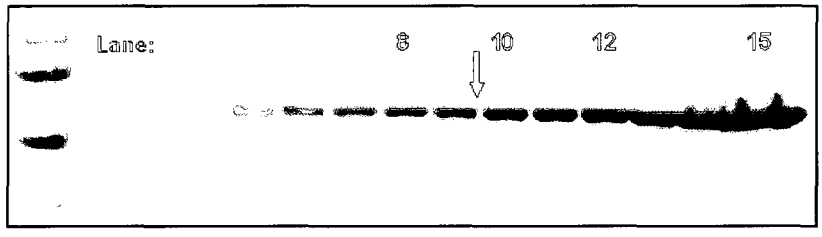



**Fig. 2.6.2.:** two-fold dilution series (lanes 8, 10, 12, and 15 are 1.5-fold) of rat brain homogenate loaded onto 10% SDS polyacrylamide gel, blotted onto PVDF membrane and probed with anti-β-tubulin antibody. Red arrow indicates band intensity selected for study of this particular antigen.

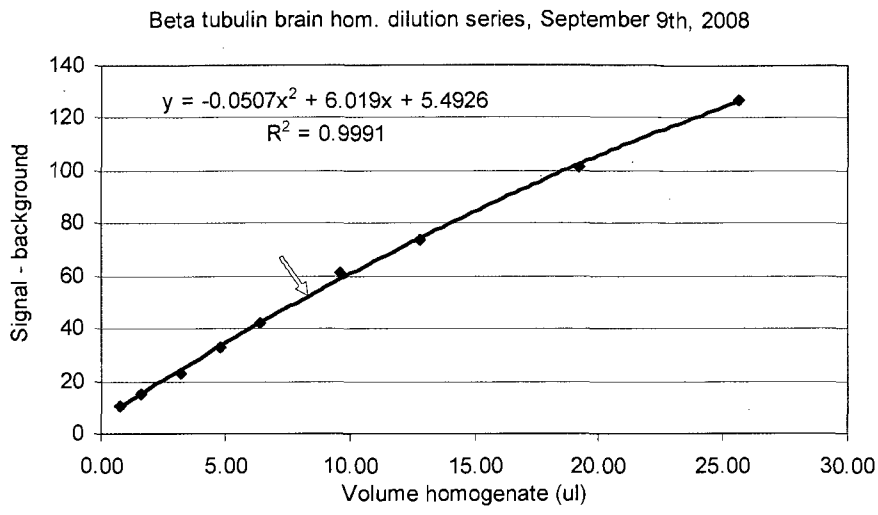



**Fig. 2.6.3.**: plot of background-subtracted densitometric signal for β-tubulin immunoreactive density and volume of homogenate applied into each lane. Red arrow indicates optimal volume of homogenate used from the experimental samples to measure this particular antigen.

The following primary antibodies were used in 3% BSA/TBST: rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal immunoglobulins (1:2,000 Z0334, DakoCytomation) as well as rabbit anti-myelin basic protein (MBP) polyclonal (1:3,000 AB980 Chemicon), mouse anti-proteolipid protein (PLP) (1:750 clone MAB388 Chemicon), mouse anti-glutamine synthetase (1:2,000 MAB302 Chemicon), rabbit anti-glutaminase (1:1000, kind gift from Dr. Norm Curthoys), mouse anti-actin (1:2,700 clone C4 Chemicon), and mouse anti-β-tubulin class III (1:830, clone TU-20 Chemicon), mouse anti-neurofilament light (NF-L) (1:1,000 Clone NR4, Sigma), mouse anti-neurofilament medium (NF-M) (1:1,000 Clone NN18, Sigma), mouse anti-α-tubulin (1:2,000 clone DM1A, Upstate), and mouse anti-β-tubulin (1:1,750 clone AA2, Upstate), mouse anti-PSD-95 (1:1,000 NeuroMab cat. # 75-028), mouse anti-syntaxin (1:5000, Synaptic Systems cat. # 110011, kind gift from Dr. Mike Tamkun), mouse anti-SNAP-25 (1:5000, Synaptic Systems cat. # 111001, kind gift from Dr. Mike Tamkun), mouse anti-total tau (1:2000 BD Transduction Laboratories cat. # 610672), mouse anti-pSer262/pSer356 tau (clone

12E8, 1 µg/ml, gift from Dr. Peter Seubert, Elan Pharmaceuticals), mouse anti-pSer396/pSer404 tau (clone PHF-1, 1:500, gift from Dr. Peter Davies). Tissue specificity studies (15 µg/lane non-diabetic rat brain, spinal cord, liver, intestine, heart and kidney) were conducted with the same dilution of primary antibodies used in the Western blot studies (see Results). Goat anti-rabbit or anti-mouse IgG coupled to AlexaFluor680 (Invitrogen) were diluted 1:10,000 in 3% BSA/TBST and used as secondary antibodies. The Odyssey InfraRed Imaging System (LI-COR Biosciences) with signal intensities that are linear up to five orders of magnitude was used to capture images and analyze results. Since Odyssey operates in the infrared spectrum, membrane autofluorescence is substantially reduced, which improves the signal-to-background ratio. The average background from at least four equal size areas from gel lanes was subtracted from the signal from each band of interest. For example, for a sample run on gel 2, the sample value per brain was calculated as (sample signal intensity – avg. membrane 2 background) x [(avg. Non-D normalization standards on membrane 1)/(avg. Non-D normalization standards on membrane 2)] x (total brain homogenate volume)/(sample homogenate volume). The results from two or more replicate electrophoresis runs were averaged, and values were reported as relative group means per brain ± s.e.m.

## 2.7. IMMUNOHISTOCHEMISTRY

At the end of 12 weeks, a separate group of rats was deeply anesthetized with ketamine/xylazine, then subjected to transcardiac perfusion with 0.9% saline to remove blood from tissues, followed by ice-cold 4% paraformaldehyde in 0.1 M PBS. Following a mid-line sagittal cut and coronal division into 4 mm blocks using a rat brain matrix (Kent Scientific, Torrington, CT), the fragments were stored in cold 4% paraformaldehyde in 0.1 M PBS for 2 months, in cold 70% ethanol for 2 months, then in

1% paraformaldehyde until used. A rat brain stereotaxic atlas was used for survey purposes.

Vibratome 50 µm floating coronal hemi-sections from rat groups were obtained from a region proximal to the bregma and incubated with the same primary antibody used for the Western blots, then processed simultaneously. Sections were incubated for 30 min in 5% normal goat serum for rabbit primary antibodies or 5% normal horse serum for mouse primary antibodies in phosphate buffered saline (PBS, Sigma) containing 0.3% Triton X-100 and 3% peroxide to quench the endogenous peroxidase. Primary antibodies were used at the following dilutions: anti-GFAP (1:1,000), anti-PLP (1:3,000), anti β-tubulin class III (1:4,000) and anti-NF-M (1:2,000). Sections were incubated in the primary antibody diluted in PBS overnight at room temperature with agitation. On the following day, slices were washed with PBS, then incubated for 1 h in biotinylated goat anti-rabbit or horse anti-mouse IgG (Vector Laboratories) diluted 1:200 in PBS. Sections were washed again in PBS and further incubated for 1 h with avidin-biotinylated peroxidase (ABC Reagent, Vectastain Elite kit). Following a thorough rinse in PBS and primary antibody binding to the antigen, a stoichiometrically pre-mixed solution composed of the secondary antibody conjugated to avidin and horseradish peroxidase (HRP) conjugated to biotin was added (see figure 2.7.1.). The signal was produced upon addition of diaminobenzidine, which is oxidized by HRP to produce an insoluble brown precipitate. The DAB reaction product is discretely localized at HRP-labeled sites,

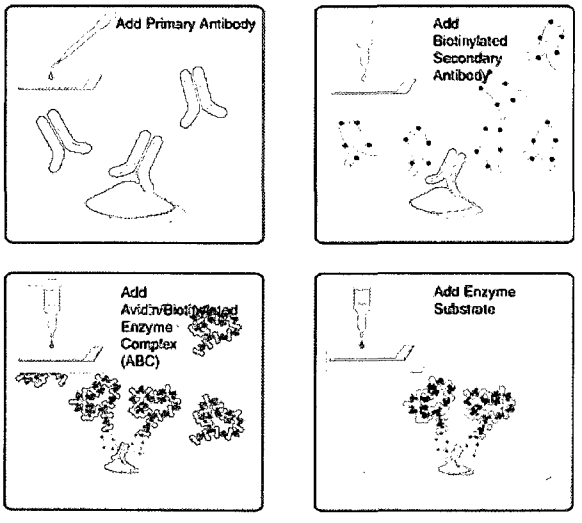



**Fig. 2.7.1.**: procedure for ABC Vectastain Elite kit, Vector Labs.

providing high resolution images of cellular antigen distribution. Advantages of this method include the virtually irreversible avidin-biotin binding and the ability to enhance the signal, since avidin has four biotin binding sites to interact with avidin and multiple HRP enzymes can be conjugated to a single biotin molecule. In addition, unlike other IHC methods involving fluorophores, the precipitate that forms does not photobleach or decay over time, making the staining permanent. After a final wash in PBS, slices were mounted on Superfrost Plus slides, air-dried, cleared in xylene and cover-slip added using Accumount 60 (Baxter Scientific Products, McGraw Park, IL). Slides were observed under brightfield illumination with neutral density filters 6 and 12 under a 40x objective using a Zeiss Axioplan 2 microscope, and images of areas of interest were captured at the same illumination using an AxioCam CCD camera and AxioVision v4.6 software (Carl Zeiss, Inc., Thornwood, NY). Images were processed and figures assembled using Adobe Photoshop 6.0 software.

Although this method is quantifiable (Benno et al., 1982), it is widely used for qualitative comparisons between groups of samples prepared and treated in parallel together. For each antigen investigated, through visual observation we empirically determined the optimal concentration of DAB, time of incubation in DAB, and antibody dilution. The relatively thick Vibratome slices permitted survey of a good volume and representative data are shown taken generally from 3-4 slices.

## 2.8. INSULIN ELISA

Two different monoclonal antibodies, each directed at a separate site on the human insulin molecule, were used in the sandwich Ultrasensitive Insulin ELISA test that used 3,3'-5,5' tetramethylbenzidine as the peroxidase substrate (Mercodia AB, Sweden). Cross-reactivity against C-peptide <0.01%, proinsulin <0.01%, hIGF-I <0.02%, hIGF-II <0.02%, and rat insulin <0.7%. Rat CSF samples were diluted 1:40 prior to test

according to manufacturer's specifications. OD450 nm was measured in a 96 microwell plate reader (Bio-Rad).

## 2.9. STATISTICAL ANALYSIS

A software program was used for statistical analysis (SAS V9.1.3). For purposes of comparing multiple group means, an ANOVA Tukey-Kramer posthoc two-tailed test was used, and significance was accepted at $p<0.05$. The values are group means ± s.e.m.

Clarification of statistical markers. * shows $p<0.05$ for Non-D *vs.* any diabetic group; † shows $p<0.05$ for D+aCSF *vs.* D+ins or D+ins+IGF; ‡ shows $p<0.05$ for D+ins+IGF *vs.* Non-D. It should be noted that where p-values are relatively close to 0.05, it is possible that significance would be achieved by addition of a few more animals. The term "statistical trend" is used herein to note these situations and exact p-values are summarized in tables 3.1.1 and 3.2.1.

# PART 3. RESULTS

## SECTION 3.1. EFFECTS OF INSULIN AND ITS COMBINATION WITH IGF ON BRAIN ATROPHY IN DIABETES – THE "12 WEEK INSULIN" EXPERIMENT

Adult rats were randomly assigned to treatment groups. Some rats were rendered diabetic with STZ and implanted with pumps that delivered either insulin (D+ins), insulin plus IGF (D+ins+IGF, also referred to as combination-treated), or artificial cerebrospinal fluid (D+aCSF) into lateral brain ventricles for 12 weeks. Untreated non-diabetic rats served as a control group (Non-D). We first tested whether these treatments prevented brain atrophy, measured as the loss of brain wet, water and dry weights as well as loss of total brain DNA and protein. These data were desired to provide baseline information concerning the magnitude of losses in these parameters at the whole brain level in diabetes, as well as magnitude of treatment effects. Table 3.1.1 at the end of this section summarizes all p-values between treatment groups represented in the Figures.

### 3.1.1. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON BRAIN WET, WATER, AND DRY WEIGHTS

Brain Wet Weight. Procedures such as TUNEL, Western blots, and Immunohistochemistry generally cannot reveal the magnitude of brain atrophy. Brain mass was directly measured by gravimetric analysis. Figure 3.1.1 shows brain wet weights for the four rat groups in the "12 week insulin" experiment, as well as p-values between groups. Brains from Non-D rats weighed 2.04 ± 0.04 g, while the brain mass

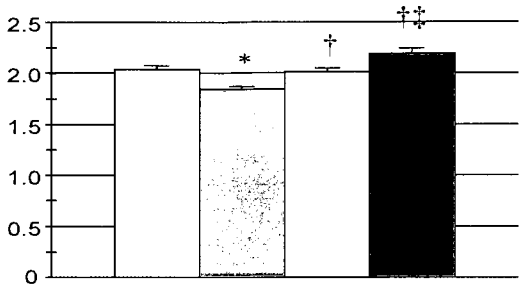



**Fig. 3.1.1.** Brain wet weight (g). All error bars are standard error of the mean (SEM). Please refer to Section 2.9 for clarification of statistical merkers used throughout this dissertation.

☐ Non-D (n=8)
☐ D+aCSF (n=9)
☐ D+ins (n=9)
■ D+ins+IGF (n=9)

from D+aCSF rats was significantly smaller by about 10%. The reduction of this magnitude is consistent with previous data from our lab (Lupien et al., 2006). Continuous insulin treatment of diabetic rats directly into the lateral brain ventricle completely prevented the loss of total brain mass, and combined treatment with IGF had a significantly greater effect than insulin alone. However, average brain mass from the combination-treated group was not significantly greater than that from the Non-D group, although there was a strong trend.

Brain Water and Dry Weights. The decrease in brain wet weight may be due to dehydration in diabetes, loss of brain cellular matter, or both. Brain homogenates were prepared from these rats (see Materials and Methods). An aliquot of brain homogenate was lyophilized in duplicate to determine brain water content and dry weight. Relative dry and water weights per brain are shown in figures 3.1.2 and 3.1.3. Brain water weight comprised 79% of total wet weight in all four groups. In brains from D+aCSF rats, both dry and water weights were significantly reduced by approximately 10% in comparison with Non-D rats, showing that brain atrophy is due to a proportional loss of water and cellular

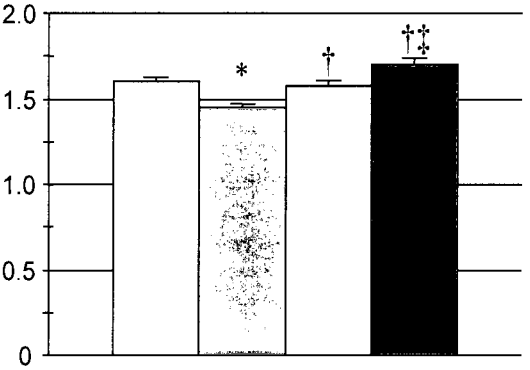



**Fig. 3.1.2.** Brain water weight (g).

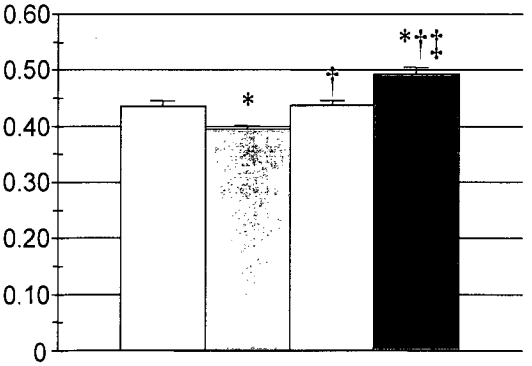



**Fig. 3.1.3.** Brain dry weight (g).

matter. Treatment with insulin completely prevented the reduction in both parameters and insulin's combination with IGF-I had a significantly greater effect than insulin alone. Brain dry weight from the combination-treated group was significantly higher compared to all other groups.

### 3.1.2. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN DNA AND PROTEIN CONTENTS

DNA Content per Brain. Brain atrophy may or may not involve loss of cells. To determine whether there was cell loss in brains of diabetic rats and whether treatments ameliorated such loss, aliquots of brain homogenates were used to measure the relative DNA content per brain using fluorometry. This method can show the magnitude of cell loss throughout the brain, whereas procedures such as TUNEL are generally more useful for studying relative regional differences (see Discussion, p. 100). Consistent with previous data reported by (Lupien et al., 2006), D+aCSF rat brains had significantly less DNA by approximately 10% compared to Non-D rats (Fig. 3.1.4). Since the amount of DNA per cell is essentially constant in the adult brain, these findings show that brain degeneration in diabetes involves cell loss of approximately 10%. Insulin treatment did not have a significant effect on that loss. On the other hand, insulin's combination with IGF-I not only prevented cell loss in diabetes, but resulted in significantly higher DNA content per brain compared to all other groups.

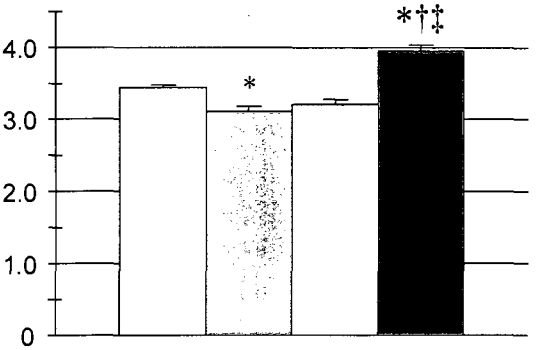



**Fig. 3.1.4.** DNA per brain (mg).

Protein Content per Brain. Previous data showed that in addition to cell loss, brain atrophy in diabetes is accompanied by a catabolic state that includes loss of total protein, a major component of brain mass (Lupien et al., 2006). There is a loss of dry weight in diabetes, and proteins comprise 56.9% of adult rat brain dry weight (Siegel, 1999). The Non-D group's mean for total brain protein was 248 mg (57% of dry weight, consistent with expectation). Figure 3.1.5 shows relative brain protein content for each treatment group in percent relative to the Non-D group mean. There was a significant decline in total brain protein content per brain in the diabetic rats, confirming previous studies. Although insulin by itself did not significantly prevent that loss, there was a strong trend and D+ins group was statistically indistinguishable from the Non-D group. Insulin's combination with IGF-I significantly and completely prevented that loss.

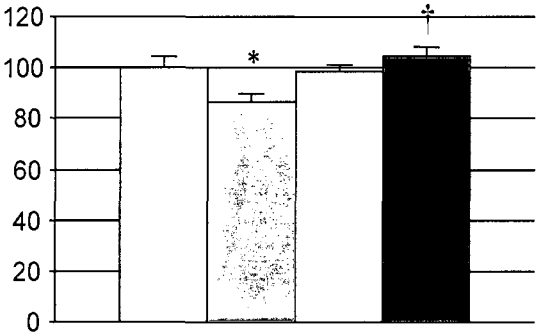


**Fig. 3.1.5.** relative brain protein (%).

Since total brain protein content was significantly reduced in brain in diabetes and since that loss contributed to brain atrophy, we next investigated relative abundances of specific proteins known to either comprise a substantial fraction of whole brain protein pool or whose selective expression is a characteristic marker for neurons or glia. Analysis of such proteins is useful because reductions in their abundance in brain in diabetes may provide insights into biochemical and physiological abnormalities that relate to brain degeneration and learning and memory impairments that accompany encephalopathy.

Western blots were used to quantitatively investigate the relative abundance of specific proteins in the brain as a whole. This was followed by immunohistochemical staining of brain sections in order to obtain a qualitative survey of the relative abundance

of some of these proteins in two brain regions considered important in learning and memory - cortex and the hippocampus. These data are described below.

### 3.1.3. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN CONTENTS OF UBIQUITOUS PROTEINS α-TUBULINS, β-TUBULINS, AND ACTINS

For Western blot studies, brains were extracted with SDS (at least 1.4 g/g protein) and urea (see Materials and Methods). Various concentrations of a clear supernatant fraction derived from whole brain homogenate were applied on gels to determine the linear range of signal intensities for each primary antibody used in tests. Detection was accomplished using a fluorescently-labeled secondary antibody; membranes were scanned with an infrared detection system that was linear over up to 5 orders of magnitude. Individual variability was determined from replicate aliquots of a single sample. Group means ± SEM showed variability between individuals within the group.

Tubulins. Tubulins are a class of highly conserved ubiquitous proteins that comprise a relatively large fraction of brain protein. Four α-tubulin genes and five β-tubulin genes are expressed in brain. Alpha- and beta-tubulin monomers form heterodimers that assemble into microtubules, cytoskeletal formations essential for a myriad of cell functions including intracellular transport, motility, mitosis, and polarity. They also contribute to the rigidity of axons and provide tracks for axonal transport.

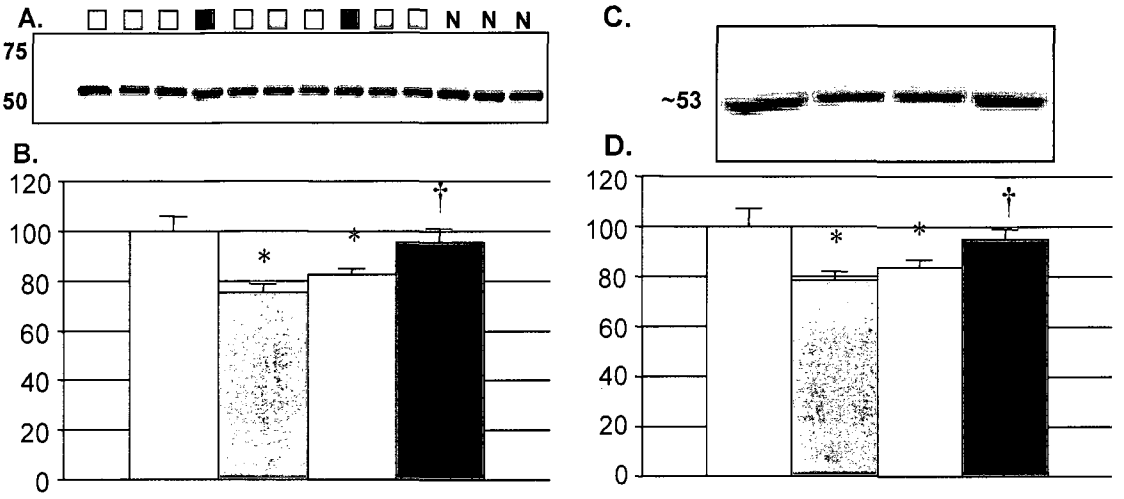



**Fig. 3.1.6. Relative α-tubulin and β-tubulin per brain.** **A.** representative Western blot membrane probed with anti-α-tubulin antibody. Color boxes above the blot represent the four experimental groups and the alternating order in which they were applied. Four membranes were used in a single experiment to accommodate all homogenates at least once and "N" represents a sample used to normalize between membranes for any inter-membrane signal differences. **B.** pooled results of two Western blot experiments showing relative α-tubulin per brain with Non-D mean set to 100%. **C.** four representative lanes from Western blot membrane probed with anti-β-tubulin antibody. **D.** pooled results of two Western blot experiments showing relative β-tubulin per brain with Non-D mean set to 100%. All error bars are SEM.

Products of the two tubulin gene families were investigated separately using antibodies raised against amino acid sequences specific for α-tubulins or β-tubulins, respectively. As expected, both antibodies revealed a single band of ~53 kDa (Fig. 3.1.6.). The average densitometric value of the Non-D group was set to 100% and mean values from other groups are reported relative to that number. Both classes of tubulins were measured in duplicate and the combined data are reported in figure 3.1.6. A and B for α-tubulins and figure 3.1.6. C and D for β-tubulins. There was a significant decrease in relative levels of α-tubulins as well as β-tubulins per brain in diabetes. Treatment with insulin alone did not have a significant effect on that decrease in either case. However, the combination of insulin and IGF-I significantly and nearly completely prevented the decline in relative abundances of both.

Actins. A large fraction of the total protein pool is comprised of cytoskeletal proteins such as actins. Mammals have at least 6 different actin isoforms, each encoded by a different gene. All 6 actins are members of a family of highly conserved

proteins which can be divided into 3 classes depending on their isoelectric point: alpha, beta, and gamma. Although the amino acid sequences of the actin isoforms are highly similar, they cannot substitute for one another *in vivo*. Their cellular roles are wide-ranging and diverse, ranging from cytoskeleton composition to intracellular cargo transport, cellular polarity, mitosis, membrane ruffling, and cell and growth cone motility.

For Western blots of whole brain SDS/urea supernatants we used MAB1501, a widely used pan-actin antibody which recognizes a highly conserved region and therefore does not discriminate between the different actin isoforms (Lessard, 1988). Since actin is present in virtually every cell, tissue specificity studies were not conducted using this antibody. Blots showed a single band of approximately 43 kDa at expected migration distance (Fig. 3.1.7. A). Three replicate Western runs were performed for this antigen and results from pooled data are shown (Fig. 3.1.7. B). Densitometric analysis of band intensity revealed that total actin content was significantly reduced in brains of D+aCSF vs. Non-D rats. Neither insulin nor the combination treatment had a significant effect on the relative actin levels in brain, although there was a trend for the combination-treated animals. These data show that actin levels are reduced in diabetes, but may not be directly regulated by insulin and IGFs in brain.

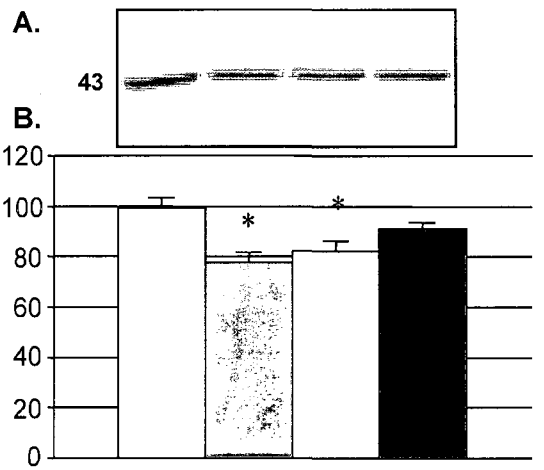



**Fig. 3.1.7. Relative actin per brain.**
**A.** Representative Western blot probed with anti-pan-actin antibody. **B.** Pooled results of three Western blot experiments showing relative actin per brain with Non-D mean set to 100%.

Actin is often used to normalize Western blots, but as shown above actin levels are not invariant in diabetes. This finding has implications where actin is used as internal control to normalize for gel loading. Since actin levels are reduced in brain in diabetes, normalizing on a "per actin" basis would make differences appear smaller than

they really are. Therefore, actin may not be an appropriate control to use in this animal model when investigating brain atrophy. These results show that a reduction in levels of high abundance proteins ubiquitous to all cells in brain may contribute to the loss of total brain protein. We next considered whether there was a reduction in proteins specific for glia and/or neurons in diabetes, and whether treatments might prevent such reductions.

### 3.1.4. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN CONTENT OF ASTROCYTE-SPECIFIC PROTEIN GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP)

Astrocytes contribute to the blood-brain-barrier by inducing and maintaining the tight junctions in cerebrovascular endothelial cells. They also envelop capillary surfaces throughout the brain, engulf nodes of Ranvier, and ensheath synapses and dendrites. To determine whether there are biochemical disturbances associated with astrocytes in diabetes, and whether treatment with insulin or its combination with IGF can ameliorate such disturbances, we measured relative content of a protein that is specifically expressed in relatively high abundance in astrocytes. Glial fibrillary acidic protein (GFAP) is an intermediate filament frequently used as a marker for astrocytes. GFAP's roles lie in maintenance of cellular mechanical strength and shape, as well as long-term upkeep of myelination. Indeed, mouse GFAP knockouts exhibit impaired myelination and deteriorated white matter (Liedtke et al., 1996).

Others have shown that GFAP immunoreactivity in brain is reduced after 8 weeks of diabetes (see Discussion, p. 105). We tested whether that reduction in GFAP levels can be prevented with insulin or combination treatments. Our Western blot specificity study detected bands of appropriate size and immunoreactivity consistent with

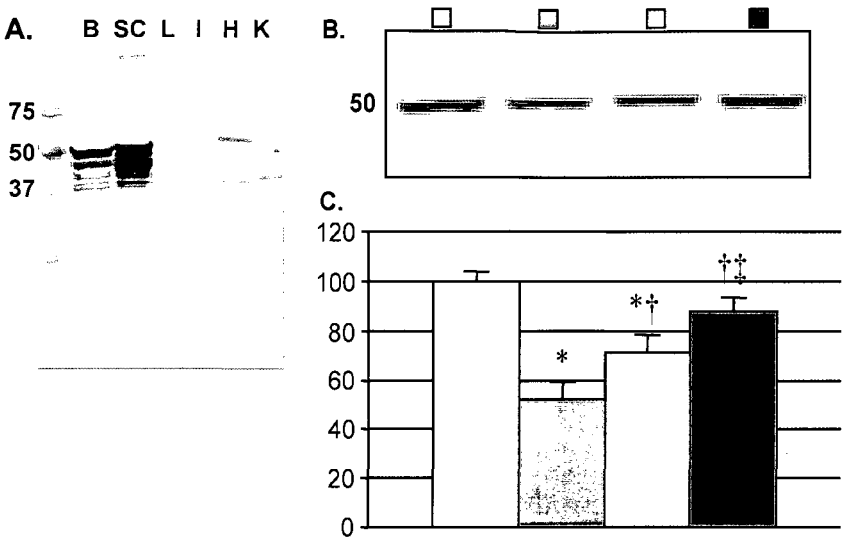



**Fig. 3.1.8. Relative GFAP per brain. A.** Specificity Western blot for GFAP using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-GFAP antibody. **C.** Pooled results of three Western blot experiments showing relative GFAP per brain with Non-D mean set to 100%.

GFAP only in brain and spinal cord, but not in liver, intestine, heart, nor kidney supernatants (Fig. 3.1.8. A). A minor band of approximately 45 kDa is believed to be a proteolytic fragment, as shown on the representative Western blot (Fig. 3.1.8. B). It comprised on average less than 3% of the total signal, hence only the main 50 kDa GFAP band was quantified. That band, in addition to other smaller bands, appears stronger in intensity in the specificity test (Fig. 3.1.8. A), since lanes were overloaded with protein. Data from three replicate Western runs were pooled and relative group mean values are shown (Fig. 3.1.8. C). Levels of GFAP in brain were significantly reduced by approximately half in D+aCSF rats relative to Non-D group, consistent with reports from others (Coleman et al., 2004; Lechuga-Sancho et al., 2006a). This reduction could be due to loss of astrocytes, reduced expression of GFAP in remaining astrocytes, or both. Insulin treatment significantly prevented that reduction, although it did not do so completely. Insulin in combination with IGF-I more completely prevented that reduction such that the D+ins+IGF group was no longer significantly different from the Non-D group.

While it was beyond the scope of this study to determine the region-specific expression of GFAP throughout brain in diabetes, we were interested to determine whether GFAP levels were reduced in two regions that are central to cognitive function. Figures 3.1.9. A and B show representative diaminobenzidine (DAB) staining corresponding to immunoreactive GFAP obtained from 50 µm coronal slices in cortex layer II and dentate gyrus of the hippocampal formation from the four experimental groups, respectively. The staining pattern in Non-D cortex was consistent with star-shaped astrocytes (arrowhead), some of which were observed to contact blood vessels with their end-feet (asterisk). Similarly, GFAP positive astrocytes were observed in both the granule cell and the molecular cell layers of the hippocampus, with particularly heavy staining in the hilus.

Both pattern and intensity of GFAP immunoreactivity in the D+aCSF sections were visibly reduced relative to Non-D tissue sections in both cortex and hippocampus (Fig. 3.1.9. A and B). Although shapes resembling astrocytes were still discernable, they were clearly more lightly stained and somewhat less abundant in diabetic slices, which may indicate loss of GFAP, loss of astrocytes, or both. By comparison, sections from combination-treated rats investigated for both brain regions were visibly more heavily stained with better discernable processes than in D+aCSF rats. While the combination-treated sections appeared similar to those from the Non-D group, sections from the D+ins group seemed to either fall between those from the Non-D and the D+aCSF groups or to more closely resemble the D+aCSF group. These results mirror the GFAP data obtained from whole brain Westerns, and show that the loss of protein in whole brain included loss of GFAP in astrocytes located in both cortex and hippocampus in diabetes.

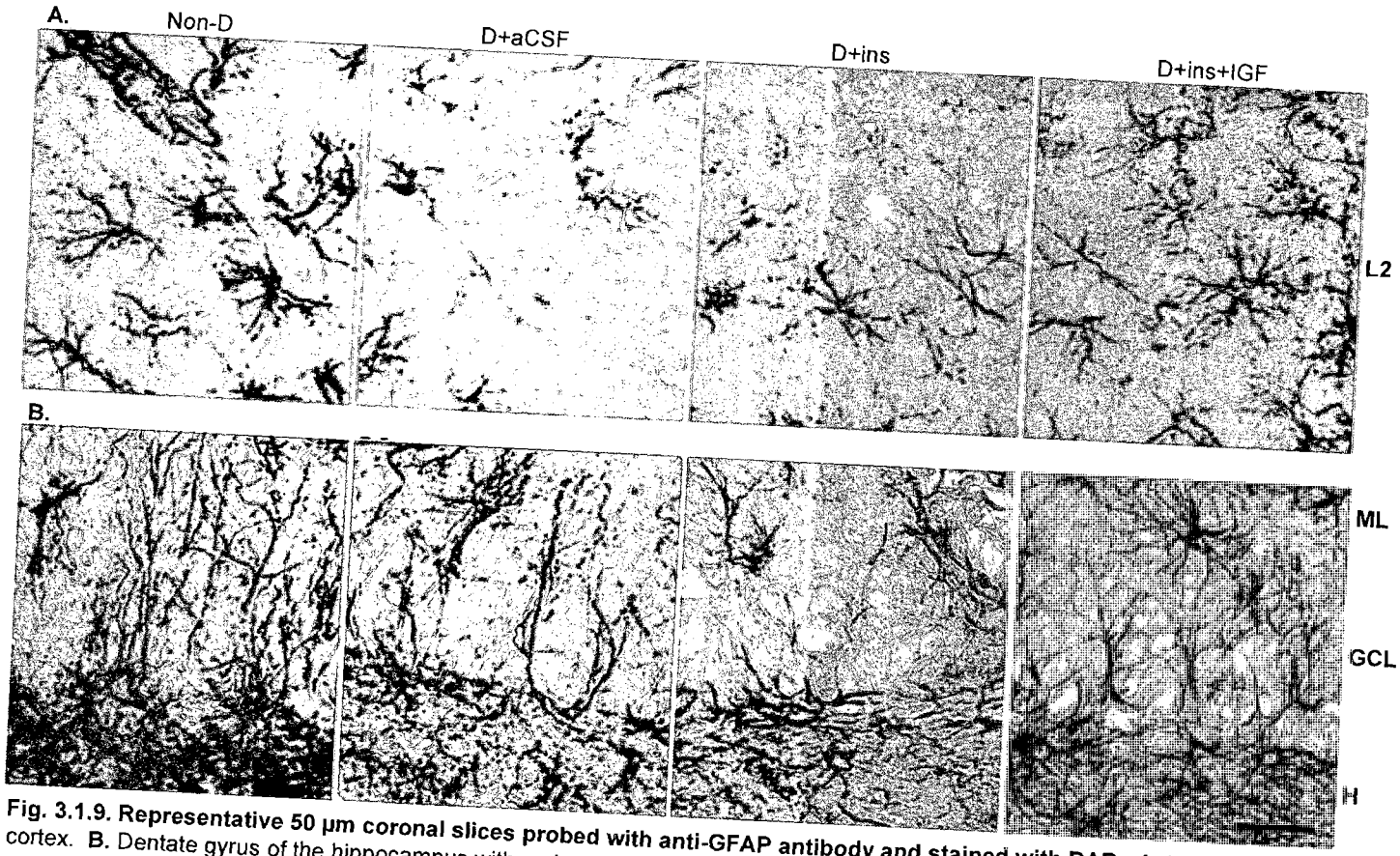



**Fig. 3.1.9. Representative 50 µm coronal slices probed with anti-GFAP antibody and stained with DAB. A.** Layer 2 (L2) of cortex. **B.** Dentate gyrus of the hippocampus with molecular layer (ML), granule cell layer (GCL), and hilus (H). Bar = 40 µm.

## 3.1.5. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN CONTENTS OF PROTEOLIPID PROTEIN (PLP) AND MYELIN BASIC PROTEIN (MBP)

Because a single oligodendrocyte can extend to and envelop as many as 50 axons, wrapping around approximately 1 mm of each and forming the myelin sheath, it may not come as a surprise that oligodendrocytes constitute 20-50% of the volume of most brain areas. Diminished myelination and impaired re-myelination after injury have been reported in diabetes, including reduced expression and abundance of myelin-specific proteins (see Introduction). To determine whether oligodendrocyte-specific proteolipid protein (PLP) and myelin basic protein (MBP) levels are reduced in brain in diabetes and whether treatment with insulin or its combination with IGF-I ameliorated such reductions, we measured relative contents of both proteins.

MBP. While PLP and MBP together make up 60 to 80% of total myelin protein in most species, MBP is estimated around 30% (Siegel, 1999). MBPs are important for stabilizing CNS myelin, as MBP-deficient mutants (the *shiverer* phenotype) exhibit delayed and reduced myelination associated with seizures, resulting in premature death. Four isoforms of MBP have been found as the result of alternative splicing of a common mRNA precursor: 21.5, 18.5, 17, and 14 kDa (Takahashi et al., 1985). Our antibody revealed four prominent bands in SDS/urea supernatants from whole brain on Western blots that had relative mobilities corresponding to the MBP isoforms (Fig. 3.1.10). A tissue specificity study indicated these bands in lanes loaded with brain or spinal cord extracts, but not liver, intestine, heart, nor kidney extracts. All four bands from brain samples were analyzed together and figure 3.1.10. C shows pooled data from two separate experiments. Relative MBP content was significantly reduced in brain in diabetes, consistent with literature. Treatment with both insulin and its combination with

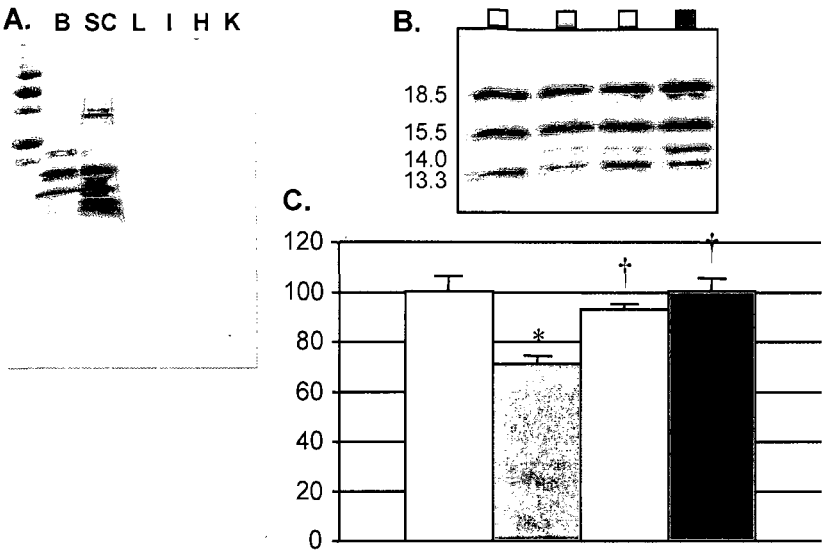



**Fig. 3.1.10. Relative MBP per brain. A.** Specificity Western blot for MBP using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-MBP antibody. Sizes of MBP splice variants are shown. **C.** Pooled results of two Western blot experiments showing relative MBP per brain with Non-D mean set to 100%. All four splice variants were quantified together.

IGF-I significantly prevented that loss; normalization was achieved because the mean values from both treatment groups were not significantly different from the non-diabetic group's mean.

PLP. PLP, also known as lipophilin, is the most abundant protein in CNS myelin (Deber and Reynolds, 1991). The amino acid sequence, strongly conserved during evolution, contains several membrane-spanning domains believed to contribute to holding the myelin layers together. There may be redundancy regarding PLP's role in CNS since myelin formation is relatively normal in PLP knockout mice, although sophisticated motor performance is affected (Klugmann et al., 1997). The molecular weight of PLP from sequence analysis is about 30 kDa, although it migrates anomalously fast on SDS gels. The *Plp* gene also gives rise to smaller splice variant DM-20, which is present in lesser abundance.

A Western blot tissue specificity test showed that our antibody detected both splice variants in samples from brain and spinal cord, but not from liver, intestine, heart,

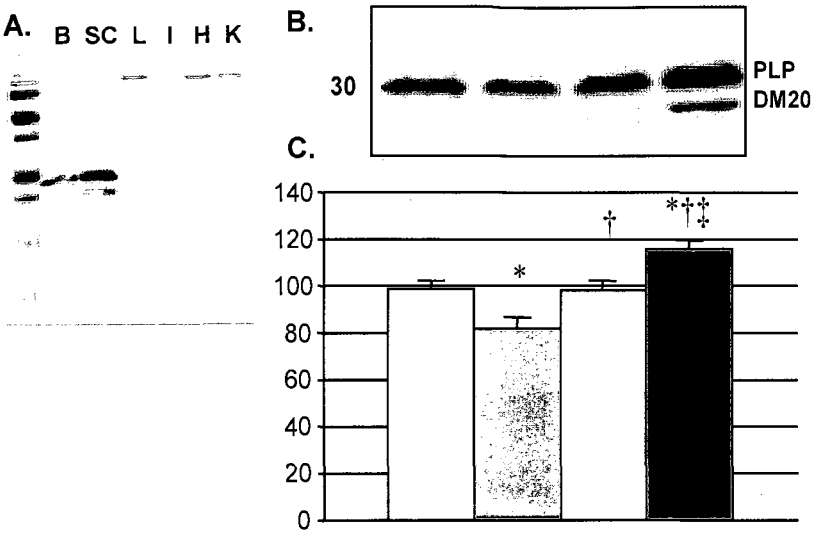



**Fig. 3.1.11. Relative PLP per brain. A.** Specificity Western blot for PLP using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-PLP antibody. PLP and minor splice variant DM20 were detected, but only PLP was quantified. **C.** Pooled results of three Western blot experiments showing relative PLP per brain with Non-D mean set to 100%.

nor kidney (Fig. 3.1.11. A). Figure 3.1.11. C shows combined data from three separate Western blot runs. DM-20 ranged between 27 and 43% of PLP band's total densitometric signal, confirming that it was present in brain in lower levels than PLP itself. However, since the role of DM-20 is not well established, we analyzed only the main PLP band intensity. PLP content in brain was significantly reduced in diabetes. Treatment with insulin completely prevented that reduction because the D+ins group's mean was statistically indistinguishable from the ND group's mean. The combination treatment also significantly prevented PLP reduction relative to the D+aCSF group, and resulted in PLP content that was highest among all groups.

To find out whether the global reduction in PLP in diabetes shown by Western blots included reductions in PLP levels in the cortex and hippocampus, and whether treatments visibly prevented these losses, we qualitatively examined the relative

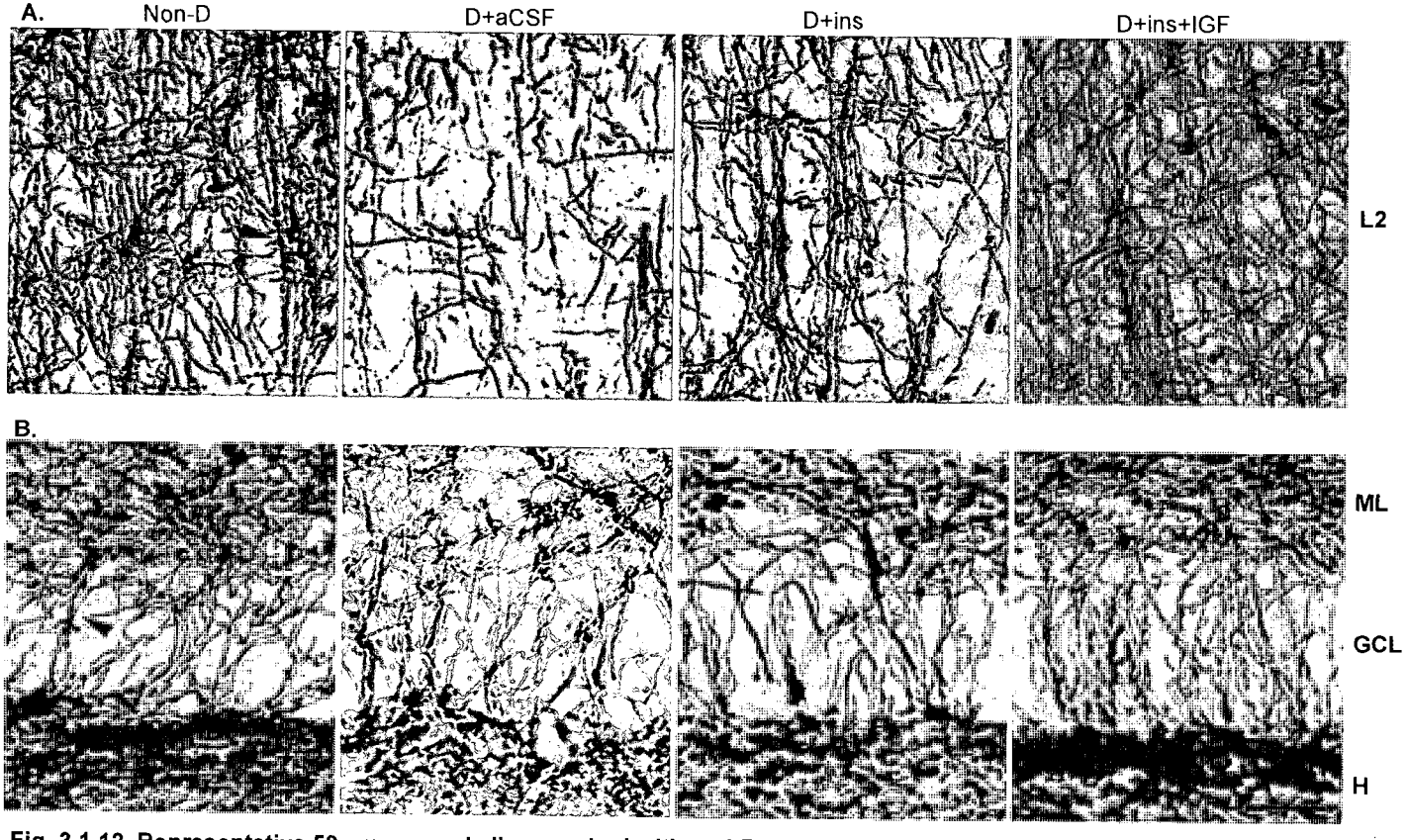



**Fig. 3.1.12. Representative 50 µm coronal slices probed with anti-PLP antibody and stained with DAB. A.** Layer 2 (L2) of cortex. **B.** Dentate gyrus of the hippocampus with molecular layer (ML), granule cell layer (GCL), and hilus (H). Bar = 40 µm.

immunoreactivity for PLP. Figs. 3.1.12 A and B show representative diaminobenzidine staining corresponding to immunoreactive PLP obtained from 50 μm coronal slices from the four experimental groups in layer II of cortex and dentate gyrus of the hippocampus, respectively. Cortex slices from Non-D rats were heavily stained in a sub-cellular architectural pattern consistent with long axonal tracts enveloped by myelin (arrowhead). In D+aCSF rats, both abundance and staining intensity were visibly reduced in cortex compared to Non-D rats. That can be attributed to reduced myelination, altered turnover of PLP, diminished number of oligodendrocytes or axons, or any combination of the above. By comparison, combination-treated rat cortices showed a highly dense network of heavily stained myelinated processes which were comparable to those observed in Non-D slices. Insulin treatment appeared to have a limited effect and more closely resembled slices from D+aCSF rats.

The dentate of the hippocampus appeared differentially affected by diabetes and treatments. Patterns resembling axonal processes ensheathed with myelin were readily observed in the granule cell layer in Non-D rats (arrowhead). However, neither the pattern nor the staining intensity were visibly different in that layer between the four groups. The same did not apply to the molecular layer and the hilus, as both the staining intensity and the pattern were visibly reduced in diabetes. Combination treatment appeared to prevent those losses, while insulin alone exhibited a partial effect. It should be noted that immunostaining included both PLP and DM-20.

Taken together, these data show that PLP content in brain in diabetes was visibly reduced in cortex as well as the molecular layer and the hilus of the hippampal formation and that the combination treatment prevented those losses, while insulin alone exhibited a limited effect. The granule cell layer was an exception, showing region-specific regulation of PLP, as PLP levels in that region appeared invariant between groups. PLP is expressed not only in myelin, but can also be present in cell bodies of

postnatal oligodendrocytes as well as neurons, including granule cells of the hippocampus (Bongarzone et al., 1999). For this reason, it is important to note that the decline in PLP immunoreactivity in diabetic rats was associated with cytoarchitecture consistent with myelinated axons.

## 3.1.6. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN CONTENTS OF GLUTAMINE SYNTHETASE (GS) AND GLUTAMINASE

Glial GS. GS is an enzyme expressed in both astrocytes and oligodendrocytes. It plays an important homeostatic role in the central nervous system by catalyzing synthesis of the amino acid glutamine from ammonia and the excitatory neurotransmitter glutamate, consuming ATP in the process:

$$\text{Glutamate} + NH_3 + \text{ATP} \rightarrow \text{Glutamine} + P_i + H_2O$$

The produced glutamine is released by glia for uptake by neurons, which convert it back to glutamate to be re-used as neurotransmitter. This process is known as the glutamate-glutamine cycle (Fig. 3.1.13). Since expression of GS is restricted to astrocytes and oligodendrocytes, measuring its relative levels may be useful to better understand not only biochemical disturbances in these two cell types, but also brain's ability to maintain the glutamate-glutamine cycle in diabetes.

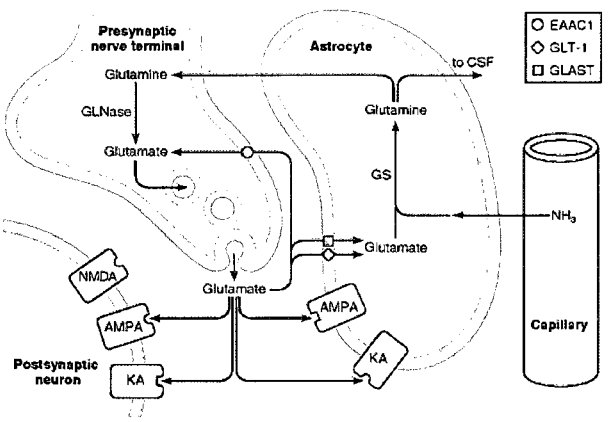



**Fig. 3.1.13. The glutamine-glutamate cycle.** Basic Neurochemistry, 6th edition.

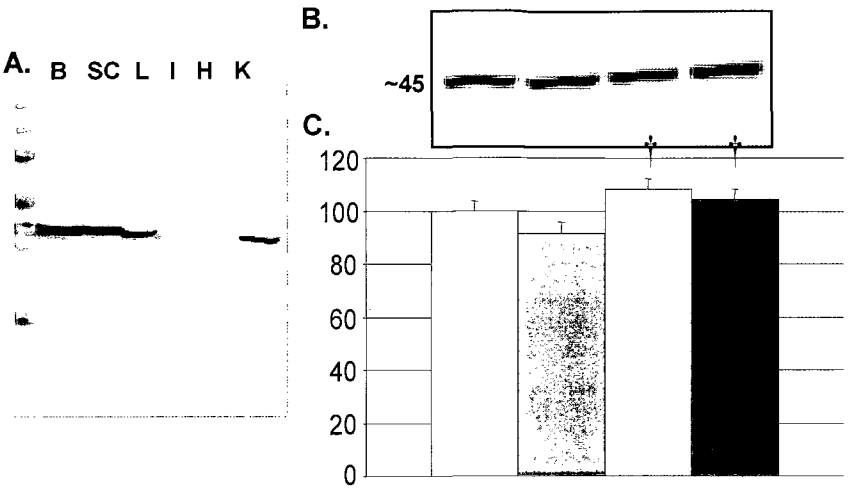



**Fig. 3.1.14. Relative glutamine synthetase (GS) per brain A.** Specificity Western blot for GS using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-GS antibody. **C.** Pooled results of two Western blot experiments showing relative GS per brain with Non-D mean set to 100%.

GS is comprised of eight identical 45 kDa subunits and our Western blots from whole brain supernatants showed a band corresponding to the expected migration distance of GS (Fig. 3.1.14 B). Consistent with expectation, the tissue specificity blot shows that GS was also expressed in spinal cord, liver, and kidney, but not in intestine and heart (Fig. 3.1.14 A). Data from two pooled Western blot experiments targeting GS showed that relative brain GS content was not significantly reduced in D+aCSF rats compared to ND group (Fig. 3.1.14 C). This shows that not all proteins are globally reduced in brain in diabetes. Treatment with insulin and its combination with IGF increased the relative abundance significantly compared to D+aCSF group, although that increase was modest.

Glutaminase. Glutaminase is glutamine synthetase's counterpart in the glutamine-glutamate cycle, responsible for conversion of glutamine to glutamate:

$$\text{Glutamine} + H_2O \rightarrow \text{Glutamate} + NH_4^+$$

Glutaminase is a mitochondrial enzyme, with at least two isoforms ubiquitously expressed in all brain regions. Its activity is high in neurons and much lower in glial cells

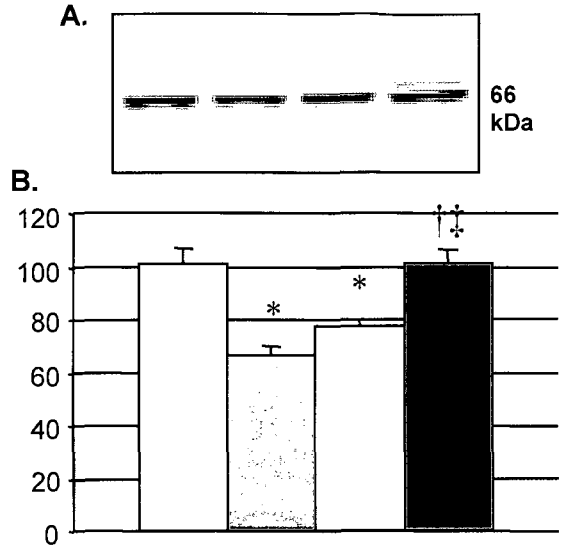



**Fig. 3.1.15. Relative glutaminase per brain.** **A.** Representative Western blot probed with anti-glutaminase antibody. **B.** Pooled results of two Western blot experiments showing relative GS per brain with Non-D mean set to 100%.

(Curthoys and Watford, 1995). We used the rabbit polyclonal antibody raised against the last ten C-terminal residues of kidney type glutaminase, a sequence which is known to be shared between human, rat, and mouse. Because glutaminase is ubiquitously expressed, tissue specificity test was not performed for this antibody. As expected, Western blots revealed a band at ~66 kDa and a fainter band ~68 kDa consistent with the longer glutaminase precursor (Fig. 3.1.15 A). Both bands were analyzed.

Combined data from two Western blot experiments revealed that glutaminase levels were significantly reduced in brains from D+aCSF animals (Fig. 3.1.15 B). Reductions in the abundance of glutaminase but not GS may contribute to an imbalance in the glutamate-glutamine cycle in diabetes. Insulin treatment did not have a significant effect on glutaminase abundance relative to D+aCSF rats, while the combination treatment with insulin and IGF-I significantly prevented reduction in diabetes and normalized the glutaminase levels.

## 3.1.7. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN CONTENTS OF NEUROFILAMENT LIGHT (NF-L), NEUROFILAMENT MEDIUM (NF-M) AND CLASS III β-TUBULIN (β-III TUBULIN)

Having found that there is loss of certain glia-specific proteins in diabetes, we next tested whether there was loss of neuron-specific proteins as well.

Neurofilaments. Neurofilaments are intermediate cytoskeletal filament proteins that determine the diameter of axons. Three types of neurofilament proteins (NF-L, NF-M, NF-H) co-assemble *in vivo*, forming heterodimers that contain NF-L plus one of the others. NF-L knockout mice lack neurofilaments and have reduced axonal diameters (Zhu et al., 1998). These mice develop normally and do not exhibit overt phenotypes other than slower rate of axonal regeneration following crush injury.

Western blot tissue specificity tests using the NF-L and NF-M antibodies

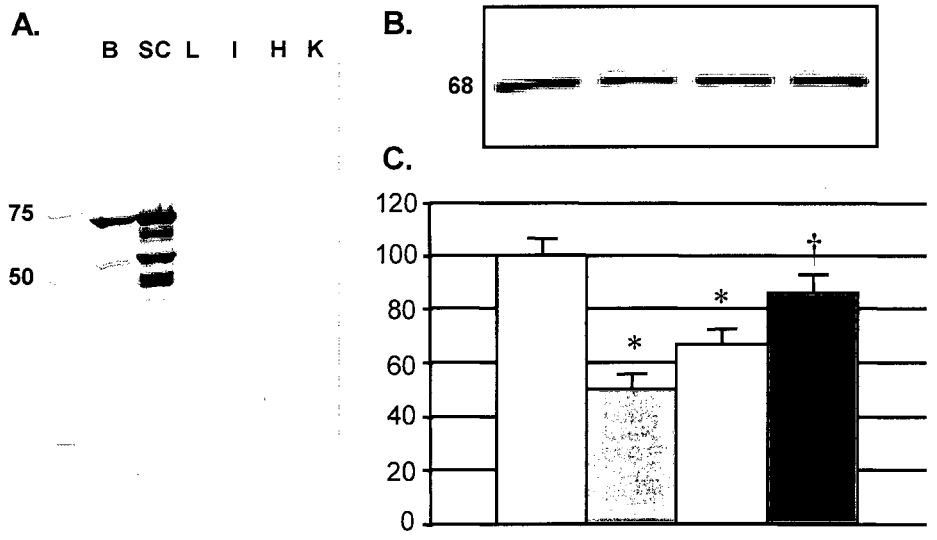



**Fig. 3.1.16. Relative neurofilament light (NF-L) per brain. A.** Specificity Western blot for NF-L using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-NF-L antibody. **C.** Pooled results of three Western blot experiments showing relative NF-L per brain with Non-D mean set to 100%.

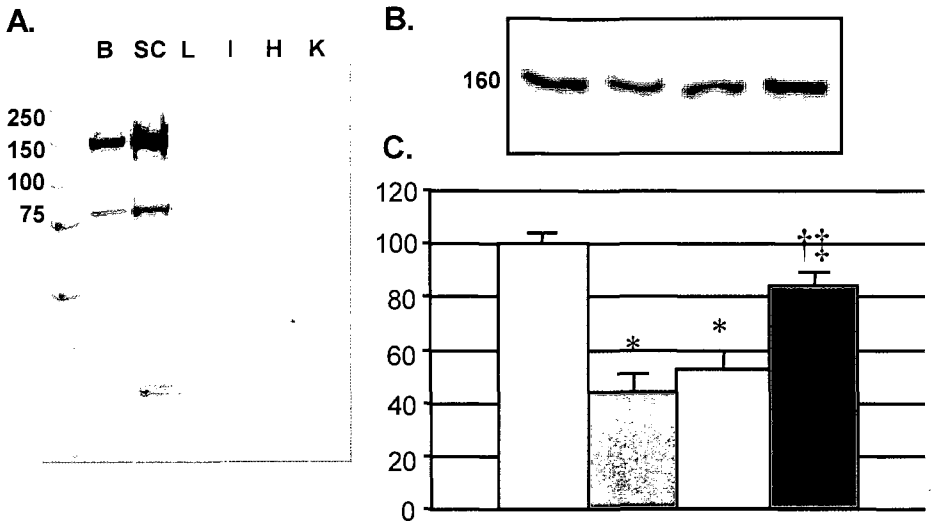



**Fig. 3.1.17. Relative neurofilament medium (NF-M) per brain. A.** Specificity Western blot for NF-L using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-NF-M antibody. **C.** Pooled results of three Western blot experiments showing relative NF-M per brain with Non-D mean set to 100%.

revealed bands at expected migration distances corresponding to 68 and 145 kDa, respectively (Fig. 3.1.16 A and 3.1.17 A). Both antibodies also revealed a few minor intensity bands of lower molecular weight in brain as well as higher intensity bands in spinal cord. The smaller bands could be attributed to proteolysis products of the two neurofilaments, or could represent other proteins with which the antibody cross-reacted. These bands were not analyzed. No prominent bands were detected in tissues from liver, intestine, heart, nor kidney at the expected migration distances.

Figures 3.1.16 and 3.1.17 show pooled data from three separate Western blot runs for NF-L and NF-M, respectively. In both cases the relative abundance of the analyzed neurofilament protein was significantly reduced in brain in abundance by about half in diabetic rats. Insulin alone did not significantly prevent these losses. On the other hand, its combination with IGF-I significantly prevented and virtually normalized these losses, as the relative mean abundance of either protein in the combination-treated group was no longer significantly lower from the Non-D group's mean. It is

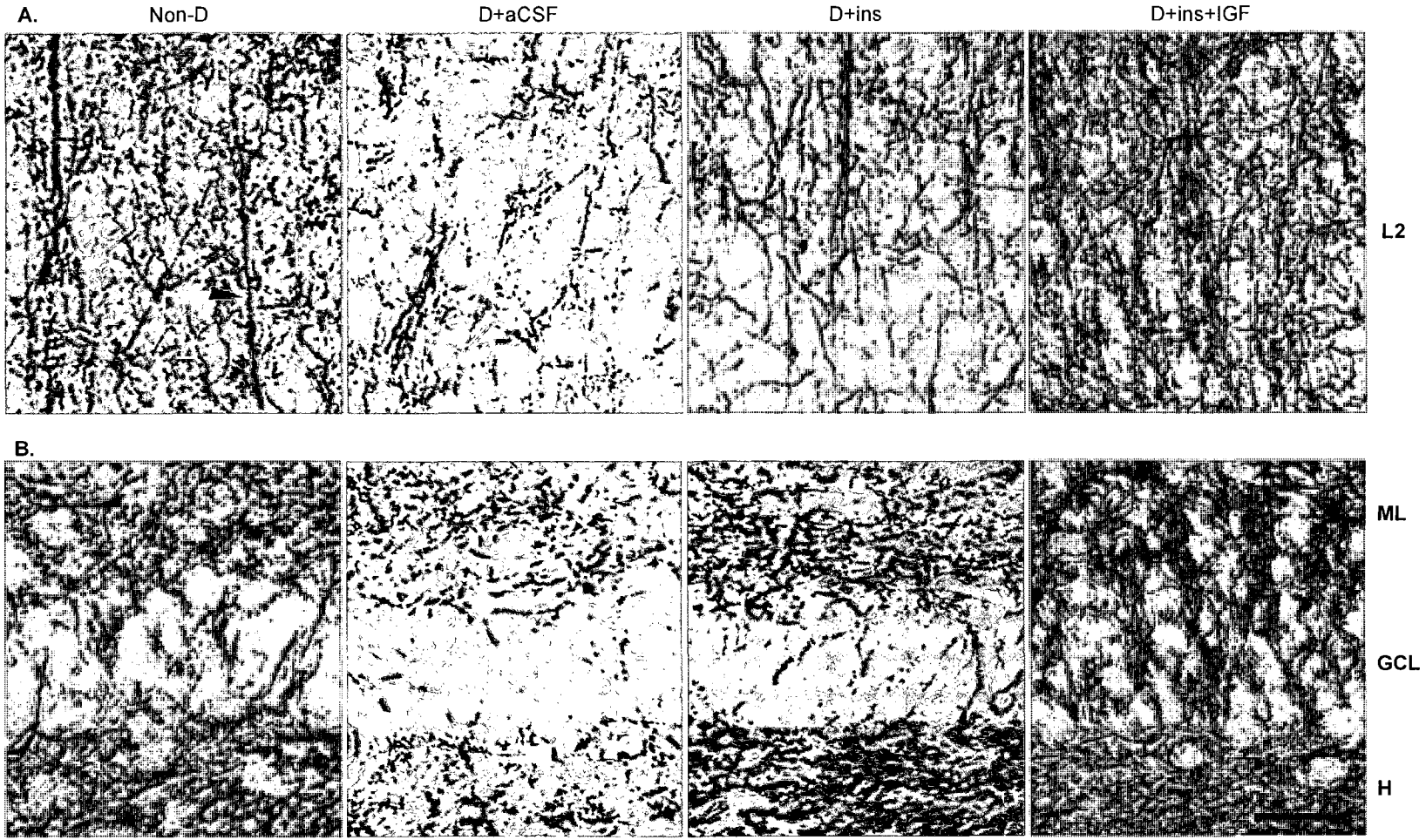



**Fig. 3.1.18. Representative 50 µm coronal slices probed with anti-NF-M antibody and stained with DAB. A.** Layer 2 (L2) of cortex. **B.** Dentate gyrus of the hippocampus with molecular layer (ML), granule cell layer (GCL), and hilus (H). Bar = 40 µm.

noticeable that both neurofilament proteins resembled one another with regard to extent of loss and prevention of that loss with treatments.

To determine whether neurons in cortex and hippocampus were included in these global losses in neurofilament levels in diabetes and whether treatments prevented them, we again turned to immunohistochemistry using NF-M as the target antigen. Fig. 3.1.18 shows representative diaminobenzidine staining corresponding to immunoreactive NF-M obtained from 50 μm coronal slices from the four experimental groups in layer II of the cortex and dentate gyrus of the hippocampus. The pattern of immunoreactive labeling in cortex resembled a network of long processes consistent with axons (see Fig. 3.1.18 A, arrowhead). The immunoreactive staining pattern in the dentate gyrus of the hippocampus was a bit more diffuse compared to the cortex, although processes could still be identified in the granule cell layer. Visually, the overall staining intensity was markedly reduced in both layer II cortex and dentate gyrus slices from D+aCSF animals. That could be attributed to either decreased abundance of NF-M per neuron, fewer neurons, fewer axons, or a combination of these possibilities. The pattern and strong intensity of immunostaining in both cortex and hippocampal slices from D+ins+IGF rats was virtually indistinguishable from the Non-D group, while insulin alone seemed to have an intermediate effect. Together, the immunohistochemical data complemented our Western blot results, showing that both layer II of cortex and dentate gyrus of the hippocampus were among brain regions that experienced losses of neuron-specific structural proteins NF-M and NF-L in diabetes, and that the combination treatment was completely effective at preventing such losses.

β-III Tubulin. Another structural protein that is found exclusively in neurons in adult mammalian brain is β-III tubulin, the only neuron-specific isoform of five β-tubulin genes (Lee et al., 1990). For our Western blot studies we used clone TU-20, an antibody that specifically recognizes class III beta tubulin and not other tubulins. A

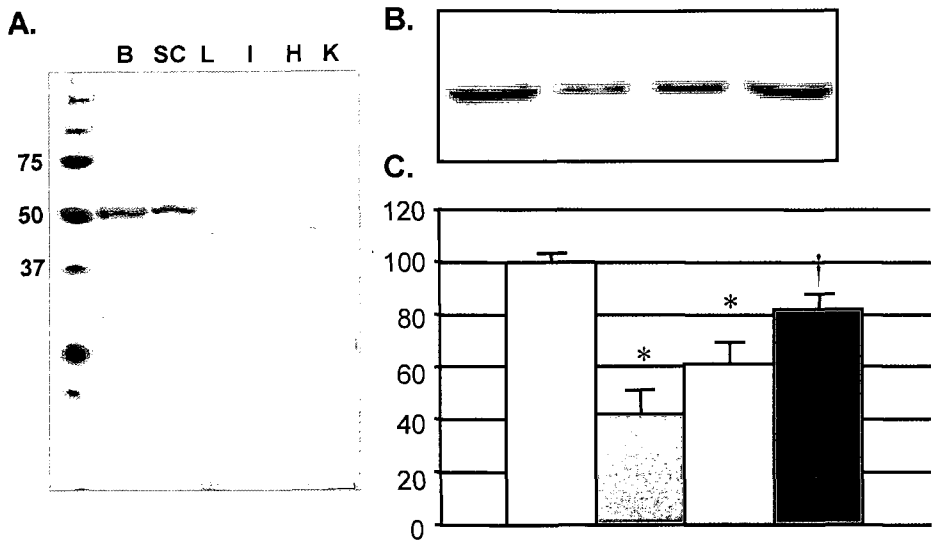



**Fig. 3.1.19. Relative β-III tubulin per brain. A.** Specificity Western blot for β-III tubulin using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-β-III tubulin antibody. **C.** Pooled results of two Western blot experiments showing relative β-III tubulin per brain with Non-D mean set to 100%.

tissue specificity test of the antibody revealed bands at expected migration distance (~50 kDa) in supernatants from both brain and spinal cord, but not from liver, intestine, heart, or kidney (Fig. 3.1.19 A). Combined Western blot data from two separate runs showed that the relative abundance of β-III tubulin was greatly and significantly reduced in diabetes (Fig. 3.1.19 C). Combination treatment with insulin and IGF-I was partially effective at preventing that global reduction, while insulin alone did not have a significant effect.

As before, with immunohistochemistry we surveyed layer II of the cortex and the hippocampus for relative β-III tubulin abundance among the four rat groups. Both brain regions layer II of the cortex and CA1 region of the hippocampus showed an immunoreactivity pattern consistent with long neuritic tracts (Fig. 3.1.20, arrowheads). The relative number of these tracts and their staining intensity was visibly reduced in both regions in D+aCSF animals. The pattern and the intensity of the staining in combination-treated rats resembled that observed with the Non-D control group. Insulin

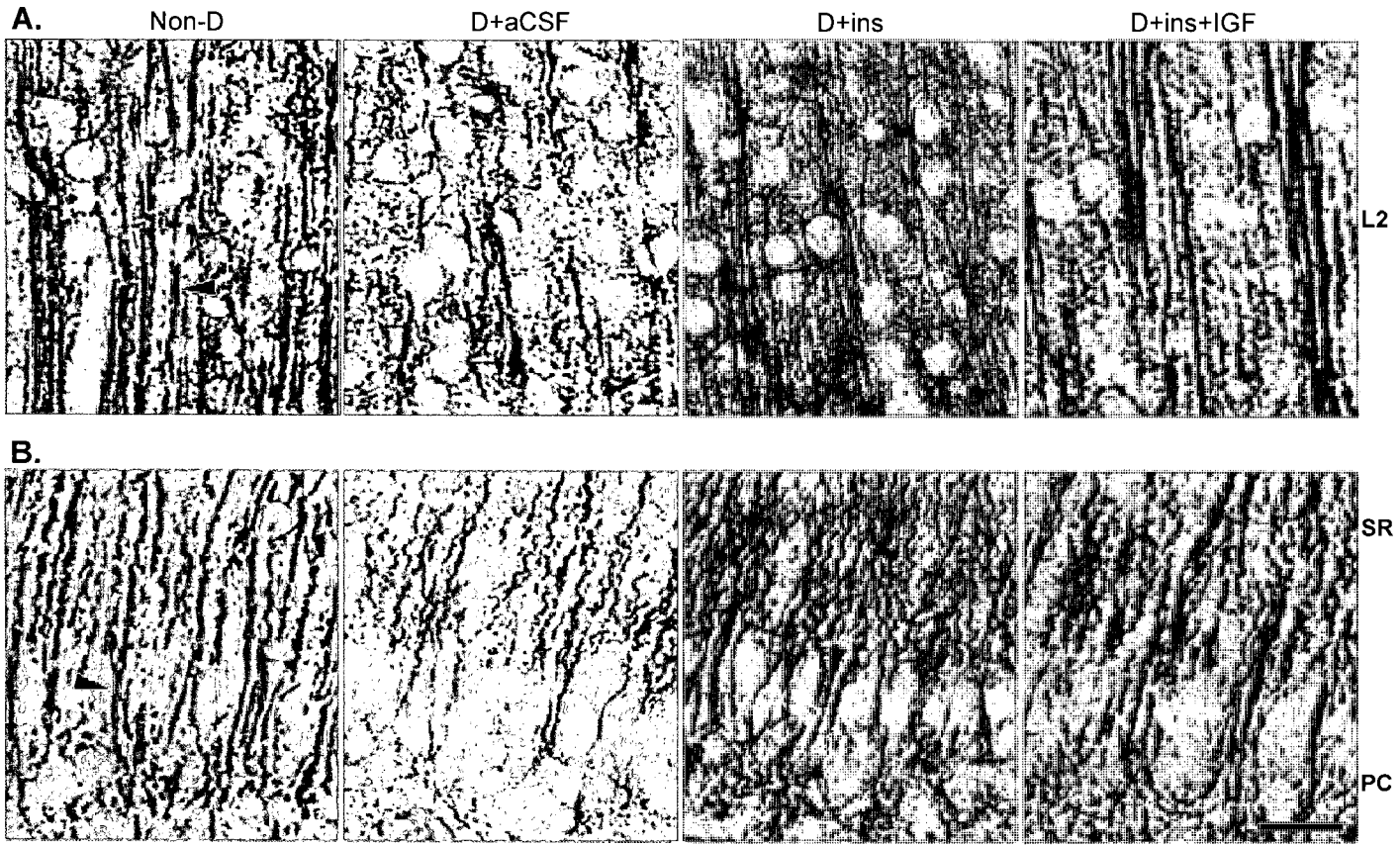



**Fig. 3.1.20. Representative 50 µm coronal slices probed with anti-β-III tubulin antibody and stained with DAB. A.** Layer 2 (L2) of cortex. **B.** CA1 region of the hippocampus with stratum radiatum (SR) and pyramidal cell layer (PC). Bar = 40 µm.

treatment did not seem to have a strong effect in preventing such loss in either region, although it appears to have been more effective in the hippocampus.

Taken together, the neuron-specific structural proteins NF-L, NF-M, β-III tubulin, and glutaminase were all greatly reduced in brain in diabetes and affected regions included the hippocampus as well as the cortex. Losses of these proteins may have important biochemical, physiological, and functional consequences, including contributions to the learning and memory deficits observed in this model and the disease (see Discussion). While insulin treatment exhibited a partial yet insignificant effects, insulin's combination with IGF-I virtually normalized the loss of all analyzed proteins. These findings indicate that these structural proteins of neurons are under the regulation of insulin and IGF, which may help prevent degeneration with learning and memory deficits observed in diabetes.

### 3.1.8. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH INSULIN OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN CONTENTS OF SYNAPSE-SPECIFIC PROTEINS SYNTAXIN, SNAP-25, AND PSD-95

Our lab and others have reported impairments in learning and memory, as well as abnormalities associated with synapses in STZ diabetic rats (see Discussion). To better understand which synapse-specific proteins may contribute to loss of synaptic plasticity and consequently learning and memory impairments in diabetes, we measured relative levels of three proteins known to be localized at pre- and postsynaptic regions of synapses. These proteins are syntaxin, SNAP-25, and PSD-95.

Syntaxin, SNAP-25, and PSD-95. Syntaxin and SNAP-25 are both SNARE (soluble N-ethylmaleimide-sensitive attachment protein receptor) proteins embedded in

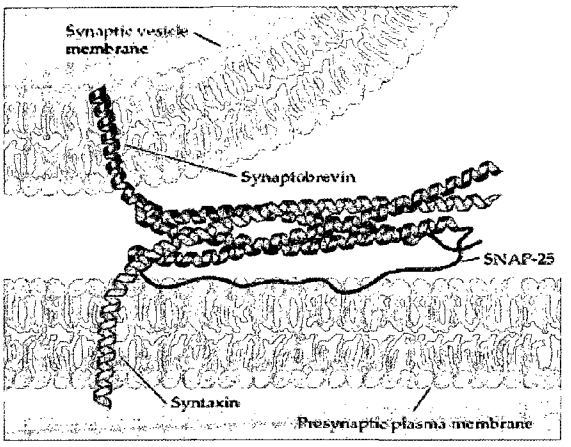



**Fig. 3.1.21. SNARE proteins.** Basic Neurochemistry, 6th edition.

the presynaptic membrane. Together with synaptobrevin, a vesicular transmembrane SNARE protein, they form a trimeric complex that primes fusion of neurotransmitter-filled vesicles (Fig. 3.1.21). Following depolarization resulting from an action potential, a large number of primed vesicles fuse with the presynaptic membrane to release neurotransmitters into the synaptic cleft. The specific role of the SNAREs in membrane fusion is still to be precisely defined and seems difficult to resolve due to the redundancy and promiscuity of SNAREs.

PSD-95 (postsynaptic density 95) is part of an electron-dense network of proteins that lies along the postsynaptic membrane. It is proposed to anchor and cluster NMDA receptors at synapses and serves as a large scaffolding molecule that links proteins and signaling molecules in and around the PSD.

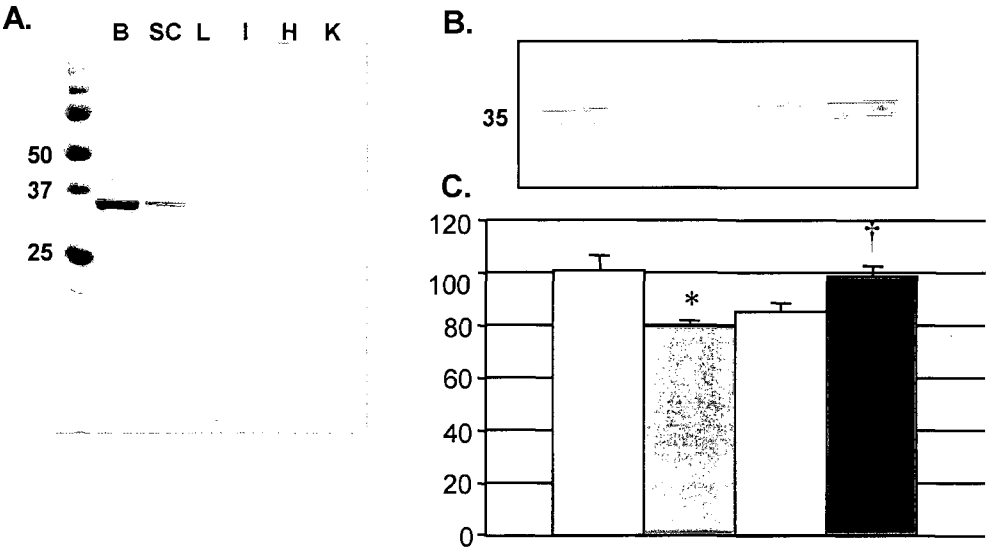



**Fig. 3.1.22. Relative syntaxin per brain. A.** Specificity Western blot for syntaxin using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-syntaxin antibody. **C.** Pooled results of two Western blot experiments showing relative syntaxin per brain with Non-D mean set to 100%.

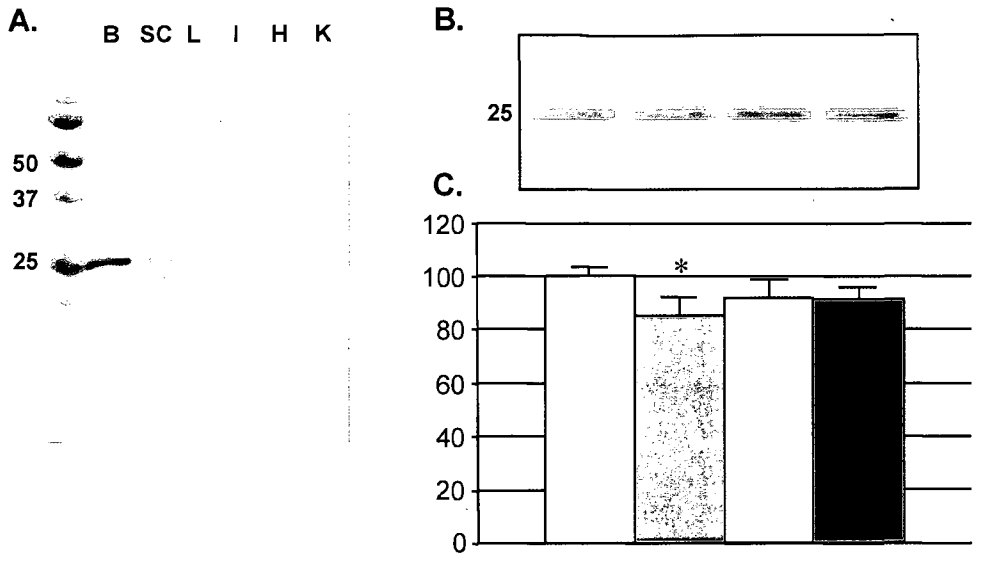



**Fig. 3.1.23. Relative SNAP-25 per brain. A.** Specificity Western blot for SNAP-25 using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-SNAP-25 antibody. **C.** Pooled results of two Western blot experiments showing relative SNAP-25 per brain with Non-D mean set to 100%.

Western blot specificity tests for syntaxin, SNAP-25, and PSD-95 revealed prominent bands at expected migration distances (35, 25, and 95 kDa, respectively) in tissues from brain and lighter bands from spinal cord, but not from liver, intestine, heart, nor kidney (figs. 3.1.22 A, 3.1.23 A, and 3.1.24 A, respectively). Pooled data from three Western blot experiments targeting syntaxin show that there was a significant decline in that SNARE protein in brain in diabetes (Fig. 3.1.22 C). Insulin treatment did not have an effect on that reduction, but insulin's combination with IGF-I completely prevented the loss.

SNAP-25 and PSD-95 were both analyzed in duplicate. Both SNAP-25 and PSD-95 relative levels were significantly reduced in diabetes (figs. 3.1.23 and 3.1.24, respectively). Interestingly, neither treatment significantly prevented the decline of either protein, although there was a slight trend for ameliorating the reduced PSD-95 abundance with the combination treatment.

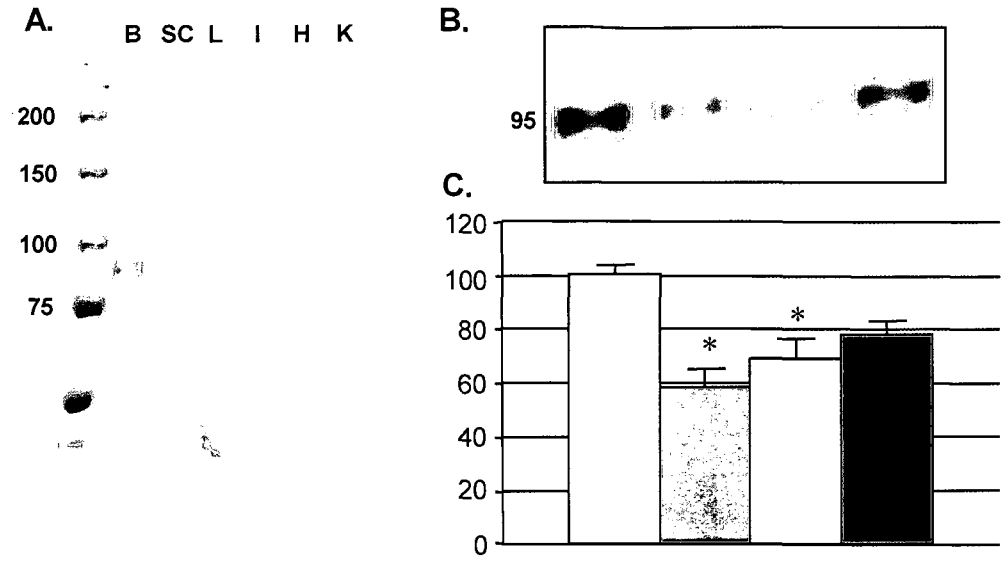



**Fig. 3.1.24. Relative PSD-95 per brain. A.** Specificity Western blot for PSD-95 using homogenates from brain (B), spinal cord (SC), liver (L), intestine (I), heart (H), and kidney (K); ~15 µg protein per lane. **B.** Representative Western blot probed with anti-PSD-95 antibody. **C.** Pooled results of two Western blot experiments showing relative PSD-95 per brain with Non-D mean set to 100%.

The data indicate that syntaxin is regulated by insulin and IGF, but SNAP-25 and PDS-95 are not. Considered together, all three synaptic proteins analyzed were significantly reduced in brain in diabetes. Losses in these proteins may represent loss of synapses, loss of neurons, reductions in expression levels of these proteins, or a combination of these possibilities. Replacement of both insulin and IGF-I may contribute to preventing loss of synapses, but further studies would be needed to determine the effect of insulin and IGF on the relative number of synapses in brain (see Discussion).

### 3.1.9. INSULIN AND IGF TREATMENTS DID NOT PREVENT HYPERGLYCEMIA NOR BODY WEIGHT LOSS IN DIABETIC RATS

The foregoing data show that insulin and IGF treatments can ameliorate or prevent brain atrophy, including loss of proteins selectively expressed in neurons or glia.

The hypothesis proposes that these treatments are effective independently of hyperglycemia. To test this hypothesis, tail blood was collected at two-week intervals over the duration of the experimental 12 weeks and glucose was measured in plasma. Plasma samples were measured in duplicate for each of the six collection days from each rat and mean plasma glucose values are shown in Fig. 3.1.25. While mean plasma glucose over 12 weeks for the Non-D group was 161 ± 16 mg/dL, D+aCSF rats were severely hyperglycemic, with group mean 554 ± 82 mg/dL. Neither insulin nor the combination treatment prevented hyperglycemia in plasma at any time over the course of the experiment. Mean values were 554 ± 60 mg/dL and 559 ± 114 mg/dL for insulin- and combination-treated animals, respectively. For all assay days there were no statistical differences between the three diabetic groups.

To exclude the possibility that hyperglycemia was affected by treatments within the brain itself, cerebrospinal fluid (CSF) was withdrawn at the *cisterna magna* prior to euthanasia and CSF glucose levels were measured. Results are shown in Fig. 3.1.26. D+aCSF-treated animals had significantly higher CSF glucose levels compared to Non-D rats: 225±17 mg/dL vs. 118±10 mg/dL, respectively. Treatment with both insulin and

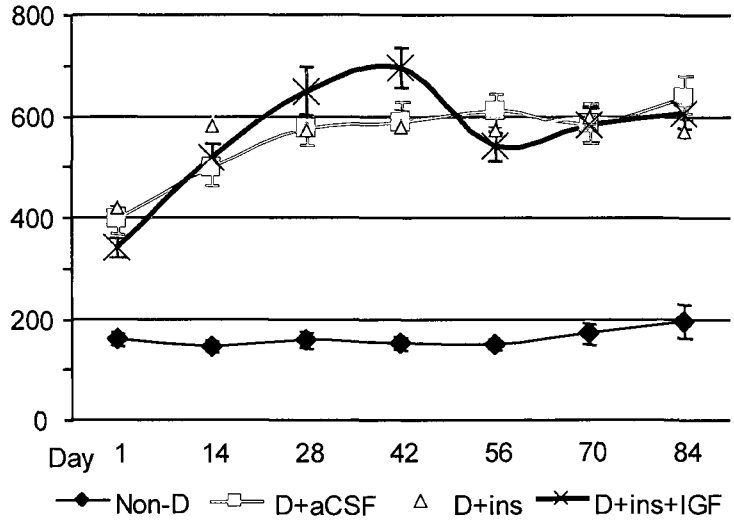



**Fig. 3.1.25.** Plasma glucose (mg/dL) throughout the "12 week insulin" experiment.

IGF-I did not significantly diminish CSF glucose (215±17 mg/dL) levels in diabetic rats. Treatment with insulin alone, however, did reduce CSF glucose significantly (200±17 mg/dL, $p<0.011$), but this decrease clearly did not prevent hyperglycemia.

**Fig. 3.1.26.** CSF glucose prior to euthanasia.

The apparent significance might be due to the smaller N size in the D+ins group, and several observations suggest that it may be a statistical anomaly. Diabetic animals enrolled in the D+ins group were receiving the same insulin dose as diabetic animals from the D+ins+IGF group, and CSF glucose was not reduced in the combination-treated animals. Neither D+ins nor D+ins+IGF reduced the plasma glucose levels, yet only in the D+ins group was CSF glucose level apparently reduced. Finally, in a separate experiment, CSF glucose levels were not reduced in D+ins vs. D+aCSF rats. Whatever the source of the apparent significance, the important point is that CSF hyperglycemia was not prevented in D+ins rats, and it seemed rather unlikely that the small decrease in CSF glucose levels could account for the actions of insulin treatment on brain atrophy.

In conclusion, all STZ-treated animals exhibited severe systemic hyperglycemia consistent with chronic depletion of insulin. Glycemic levels were also elevated in the CSF. Neither plasma nor CSF glucose levels were reduced by the combination treatment. Plasma glucose levels were not reduced by the insulin treatment, and most likely CSF glucose levels were not reduced as well.

Chronic depletion of insulin results in a catabolic state with severe weight loss. Indeed, diabetic rats from all three groups had significantly lower body weights than Non-D rats at the conclusion of the experiment (Fig. 3.1.27). Neither insulin alone nor

the combination treatment prevented the body weight loss in diabetic animals. Thus, while insulin and IGF may exit from CSF at the superior *sagittal sinus* into the blood stream, the amounts were insufficient to reduce plasma hyperglycemia nor weight loss in diabetic rats. This was not unexpected, because brain is less than 1% of body weight, and insulin and IGF concentrations would be reduced by metabolism during transit through the ventricular system as well as diluted at least 100-fold upon entering the systemic circulation.

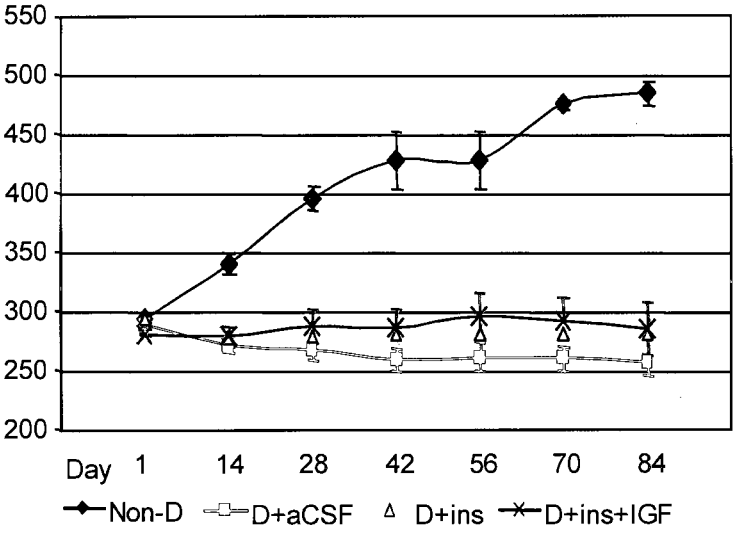



**Fig. 3.1.27.** Body weight between groups over 12 week duration of the experiment.

### 3.1.10. CSF HUMAN INSULIN CONCENTRATIONS IN DIABETIC RATS

To show that administered human insulin was indeed delivered into the CSF of animals from both D+ins and D+ins+IGF groups, we performed a sandwich ELISA on collected CSF samples. This ELISA was specific for human insulin and did not detectibly cross-react with either rat insulin nor IGFs. Our specificity test showed that the kit indeed did not cross-react with either human IGF-I nor human IGF-II (not shown). The low signal in D+aCSF CSF showed that cross-reactivity with rat insulin and IGF was

relatively minor, if any. The data showed that relative to the baseline absorbance observed in samples from Non-D and D+aCSF groups, h-insulin concentration in CSF from D+ins and D+ins+IGF rats was significantly higher (Fig. 3.1.28). This shows that insulin was indeed present in CSF following treatments.

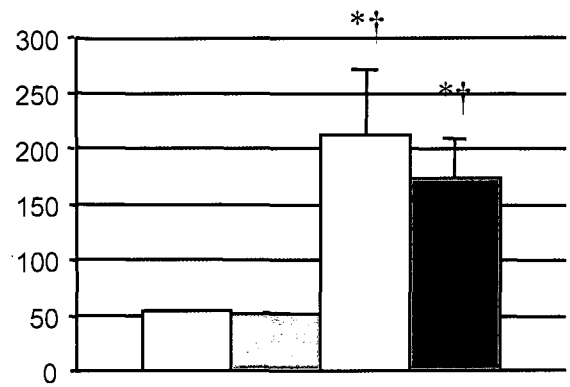


**Fig. 3.1.28.** CSF insulin (μU/mL).

Summarized (table 3.1.1 below), the data from the "12 week insulin" experiment show that insulin treatment prevented loss of brain mass and cerebral dehydration in diabetic rats. Insulin also ameliorated loss of glia-specific proteins, although the prevention of the loss was not complete. Treatment with insulin in combination with IGF-I was considerably more effective so that the decline in every measured parameter was either normalized or significantly prevented; the combination treatment completely prevented brain atrophy that included loss of cells, total protein content, ubiquitous proteins, as well as glia- and neuron-specific proteins. The treatments prevented brain atrophy despite unabated hyperglycemia and weight loss in diabetic rats. Most of these results have been accepted for publication and are now in press in *Brain Research* under the title "*Insulin and IGF-I prevent brain atrophy and DNA loss in diabetes*" with authors P. Šerbedžija, J.E. Madl, and D. N. Ishii (Serbedzija et al., 2009).

| Table 3.1.1 – Summary of statistical comparisons for the "12 week insulin" experiment | | | | |
|---|---|---|---|---|
| **Parameter** | **D+aCSF** vs. Non-D[a] | **D+aCSF** vs. D+ins vs. D+ins+IGF[b] | **D+ins+IGF** vs. D+ins[c] | **Non-D** vs. D+ins vs. D+ins+IGF[d] |
| Brain wet wt. | <0.013* | <0.025†<br><0.001† | <0.018‡ | <0.98<br><0.052 |
| Brain water wt. | <0.010* | <0.030†<br><0.001† | <0.037‡ | <0.95<br><0.14 |
| Brain dry wt. | <0.045* | <0.023†<br><0.001† | <0.003‡ | <0.99<br><0.002* |
| DNA | <0.029* | <0.78<br><0.001† | <0.001‡ | <0.20<br><0.001* |
| Total protein | <0.050* | <0.078<br><0.004† | <0.61 | <0.99<br><0.79 |
| α-tubulin | <0.001* | <0.49<br><0.002† | <0.061 | <0.009*<br><0.82 |
| β-tubulin | <0.001* | <0.76<br><0.017† | <0.15 | <0.016*<br><0.71 |
| Actin | <0.003* | <0.850<br><0.093 | <0.38 | <0.021*<br><0.44 |
| GFAP | <0.001* | <0.008†<br><0.001† | <0.024‡ | <0.001*<br><0.15 |
| MBP | <0.001* | <0.011†<br><0.001† | <0.63 | <0.64<br>1 |
| PLP | <0.040* | <0.044†<br><0.001† | <0.030‡ | 1<br><0.032* |
| GS | <0.14 | <0.001†<br><0.011† | <0.73 | <0.20<br><0.70 |
| Glutaminase | <0.001* | <0.068<br><0.001† | <0.001‡ | <0.001*<br>1 |
| NF-L | <0.001* | <0.23<br><0.002† | <0.18 | <0.006*<br><0.42 |
| NF-M | <0.001* | <0.80<br><0.001† | <0.006‡ | <0.001*<br><0.28 |
| β-III tubulin | <0.001* | <0.24<br><0.002† | <0.17 | <0.003*<br><0.32 |
| Syntaxin | <0.007* | <0.77<br><0.018† | <0.13 | <0.056<br><0.96 |
| SNAP-25 | <0.031* | <0.58<br><0.68 | 1 | <0.35<br><0.27 |
| PSD-95 | <0.001* | <0.57<br><0.13 | <0.77 | <0.010*<br><0.089 |

[a] Shows whether the parameter was significantly reduced in diabetes.
[b] Shows the effect of insulin or its combination with IGF-I on the parameter in diabetes.
[c] Shows whether insulin's combination with IGF-I was more effective than insulin alone. Notice that combo treatment exceeded the Non-D group in few cases.
Significance accepted at $p<0.05$, Tukey-Kramer *posthoc* test of means.
Statistical symbols *†‡ from table correspond to symbols in figures.
For all tests in the "12 week insulin" experiment, the n-values are as follows: Non-D 8; D+aCSF 9; D+ins 9; D+ins+IGF 9.

## SECTION 3.2. EFFECTS OF IGF AND ITS COMBINATION WITH INSULIN ON BRAIN ATROPHY IN DIABETES

The "12 week insulin" experiment left the question open whether the observed salutary effects of the combination treatment were the result of the combined action of insulin and IGF-I or due to action of IGF-I alone. For that reason, a follow-up experiment was conducted involving the same design and groups of rats except that insulin-treated group was replaced by an IGF-I treated group (D+IGF) (see Materials and Methods). The dose of IGF-I was the same as that administered in the D+ins+IGF group. This experimental design permitted testing of the hypothesis that the combination of insulin and IGF was more effective than IGF alone in preventing brain atrophy.

### 3.2.1. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH IGF-I OR INSULIN IN COMBINATION WITH IGF-I ON BRAIN WET, WATER AND DRY WEIGHTS

Brain Wet Weight. The mean brain wet weight in D+aCSF rats was 9% significantly lower compared to Non-D group, resembling data from the "12 week insulin" experiment (Fig. 3.2.1). The combination treatment once again prevented that decline.

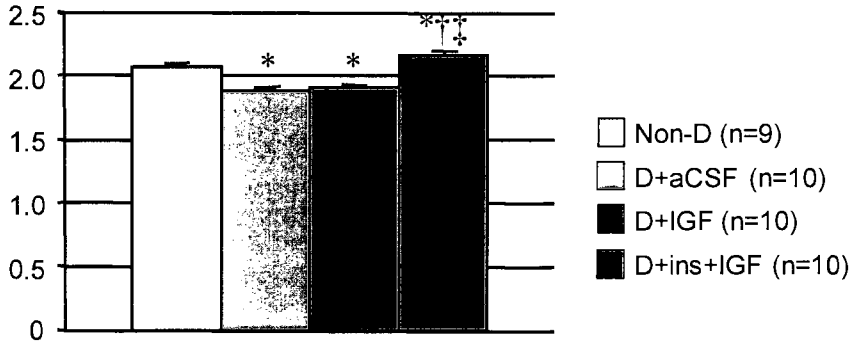



**Fig. 3.2.1.** Brain wet weights and, groups, and rat numbers enrolled in "12 week IGF" experiment. All error bars are standard error of the mean (SEM).

IGF-I alone, however, did not significantly prevent loss of brain wet weight.

Brain Dry and Water Weights. Brain homogenate aliquots were again lyophilized as in the previous experiment to determine the water and dry weights. Both water and dry weights were significantly reduced on average 9% compared to the Non-D group, again resembling the "12 week insulin" experiment data (figures 3.2.2 and 3.2.3, respectively). As expected, the combination treatment prevented the decline in both parameters. However, the mean value for the IGF-I treated group was statistically indistinguishable from the D+aCSF group, showing that IGF-I by itself did not have a detectable effect. Therefore, IGF-I did not have an effect on the loss of bulk brain mass in diabetes. The observed salutary effects of the combination treatment must therefore be due to combined actions of both insulin and IGF-I.

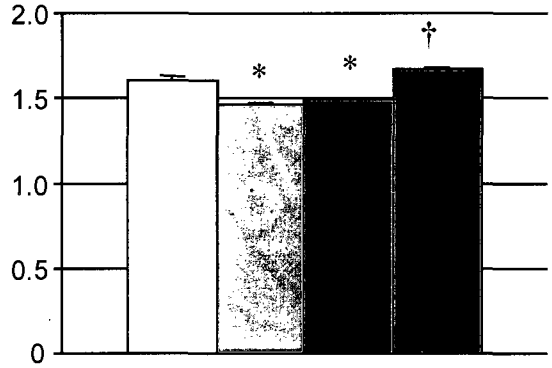



**Fig. 3.2.2.** Brain water weight (g).

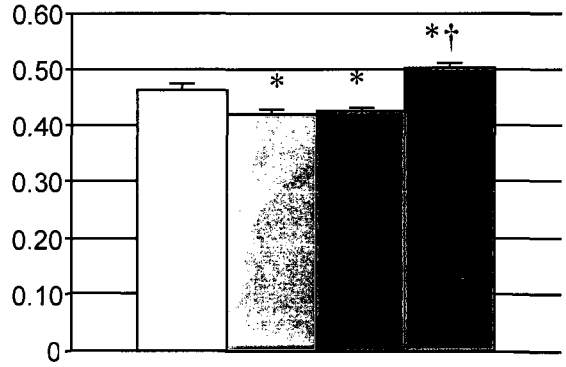



**Fig. 3.2.3.** Brain dry weight (g).

## 3.2.2. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH IGF-I OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN DNA AND PROTEIN CONTENTS

DNA Content per Brain. To determine whether IGF-I significantly prevented losses in total DNA in brain, aliquots of brain homogenate from the four groups were analyzed using fluorometry. Measurements were performed in quadruplicate for each

rat and values were back-calculated to the whole brain, as before. Two separate measurements were performed on this group of rats and both showed that there were virtually no differences in DNA content between any of the groups (not shown). This is in contrast to previous findings where DNA loss was observed in the "12 week insulin" experiment as well as previously published results (Lupien et al., 2006). However, it was anticipated that a catabolic state with protein loss would need to be of sufficient duration and diabetic intensity to progress to DNA loss. STZ strength is known to be variable in different chemical batches and it slowly oxidizes over time. Other variables may include relative health and nutritional status of different batches of rats. Hence, DNA loss may not occur in every 12 week experiment. Therefore, whether IGF-I would be able to measurably prevent brain cell loss by itself remains unknown, although it can prevent increased TUNEL staining in neuroretina (Seigel et al., 2006) (see Discussion).

Protein Content per Brain. Pilot experiments in this laboratory established that protein loss precedes DNA loss. Fig. 3.2.4 shows that brain total protein content was significantly reduced in D+aCSF animals compared to the Non-D group in the present experiment, resembling protein reduction observed in the "12 week insulin" experiment. This reinforces the hypothesis that degeneration associated with protein loss precedes loss of cells. As before, treatment with both insulin and IGF-I completely prevented that decline, but IGF-I alone was ineffective. This is in agreement with the hypothesis that the combination treatment is more effective than IGF-I alone.

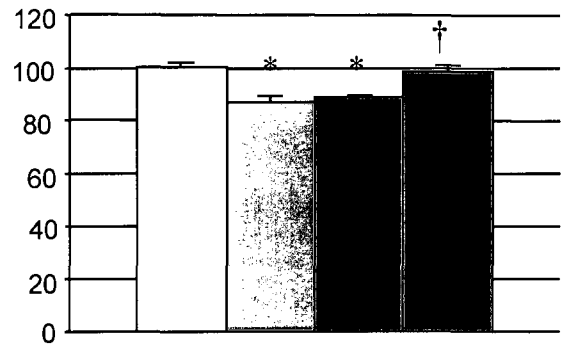



**Fig. 3.2.4.** Relative brain protein (%).

### 3.2.3. EFFECT OF DIABETES AND I.C.V. TREATMENT WITH IGF-I OR INSULIN IN COMBINATION WITH IGF-I ON RELATIVE BRAIN CONTENTS OF GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP), NEUROFILAMENT LIGHT (NF-L), AND NEUROFILAMENT MEDIUM (NF-M)

Astrocytic GFAP and neuronal neurofilaments medium and light were significantly reduced in the "12 week insulin" experiment. Although the severity of degeneration appeared attenuated in this experiment, the possibility was tested that GFAP, NF-M, and NF-L were significantly reduced in diabetes. Western blots were performed in duplicate for each antigen using the same antibodies and experimental procedures as before.

GFAP, NF-L, and NF-M contents per brain were significantly reduced in D+aCSF animals (figs. 3.2.5, 3.2.6, and 3.2.7, respectively). In all three cases, the combination treatment significantly prevented these reductions observed in D+aCSF animals, confirming our earlier results. However, IGF-I alone did not have a significant effect on

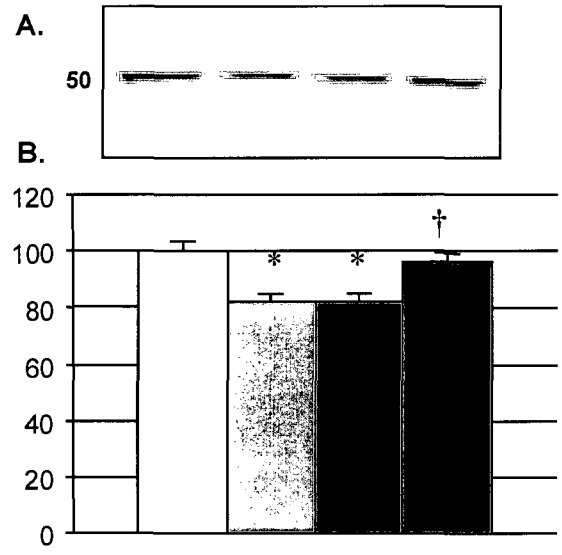



**Fig. 3.2.5. Relative GFAP per brain. A.** Representative Western blot probed with anti-GFAP antibody. **B.** Pooled results of two Western blot experiments showing relative GFAP per brain with Non-D mean set to 100%.

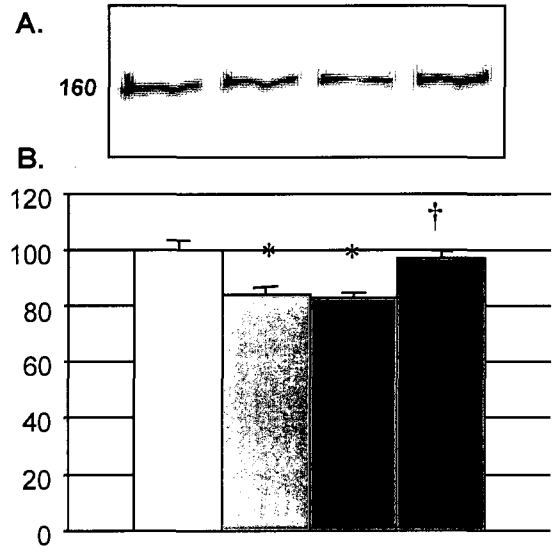



**Fig. 3.2.6. Relative NF-M per brain. A.** Representative Western blot probed with anti-NF-M antibody. **B.** Pooled results of two Western blot experiments showing relative NF-M per brain with Non-D mean set to 100%.

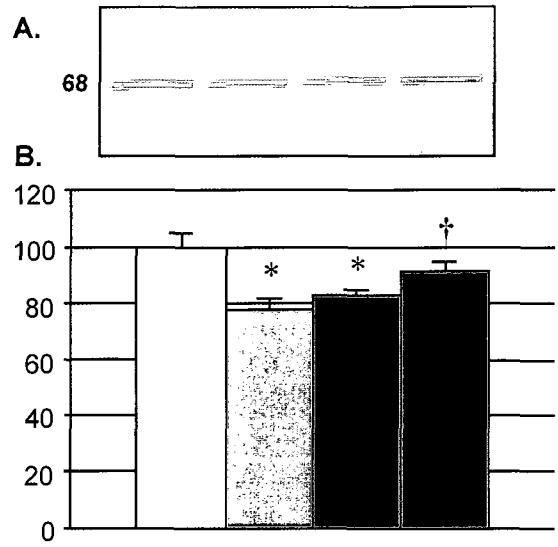



**Fig. 3.2.7. Relative NF-L per brain. A.** Representative Western blot probed with anti-NF-L antibody. **B.** Pooled results of two Western blot experiments showing relative NF-L per brain with Non-D mean set to 100%.

the decline of any of these proteins.

Taken together, IGF-I alone did not have a significant effect on any of the measured parameters where there was a significant reduction in D+aCSF animals. These results indicate that it was the combination treatment of insulin and IGF-I that was predominantly responsible for preventing brain atrophy and degeneration. The combination treatment was clearly superior to IGF-I alone in the prevention of brain atrophy.

## 3.2.4. EFFECT OF DIABETES ON RELATIVE BRAIN CONTENT AND PHOSPHORYLATION OF TAU

There are disorders other than diabetes in which brain atrophy and dementia are associated with concomitantly reduced insulin and IGF levels, such as late-onset Alzheimer's Disease (see Discussion). Hyperphosphorylation of the microtubule-associated protein tau is a hallmark feature of AD as well as several other neurodegenerative diseases, a feature collectively known as tauopathy (Lee et al., 2001). Hyperphosphorylation at certain residues abrogates the ability of tau to bind and stabilize microtubules, leading to tau accumulation as paired helical filament (PHF) and aggregation as major component of insoluble intracellular neurofibrillary tangle deposits.

Indeed, there are reports of increased phosphorylation of such residues of tau in experimental models of diabetes, including STZ-diabetes (see Discussion).

In order to better understand the biochemical pathology associated with brain atrophy in the STZ diabetic rat, we tested whether tau levels were reduced or whether tau was hyperphosphorylated at specific residues already documented in a mouse model of STZ diabetes as well as in AD. Brain SDS/urea supernatants from the "12 week IGF" experiment were resolved on SDS-PAGE and blotted in the same manner as with other investigated antigens, and membranes were probed with two monoclonal antibodies widely used for detection of tau hyperphosphorylation associated with AD. Brain homogenates contained 2 mM sodium vanadate to prevent de-phosphorylation of proteins. Antibody 12E8 detects increased phosphorylation at Ser262 in the M1 and Ser356 in the M4 microtubule binding domains of tau, a uniquely phosphorylated residue in PHF-tau (Seubert et al., 1995). Since phosphorylation of Ser262 is not sufficient to prevent tau binding to microtubules, phosphorylation at multiple sites is required. For that reason, we also investigated antibody PHF-1, an antibody raised against aggregated PHF protein (Greenberg et al., 1992) that detects increased phosphorylation at Ser396 and Ser404 in the carboxy-terminal domain of tau. While Ser262, Ser356, and Ser396 were found to be specifically hyperphosphorylated in postmortem tissues from AD patients, Ser404 was not (see Fig. 3.2.8 from (Hanger et al., 2009)).

Both antibodies specifically detect the phospho- rather than the de-phospho form of tau at the discussed amino-acid residues. In addition, total tau was quantified independently of phosphorylation status with a third antibody.

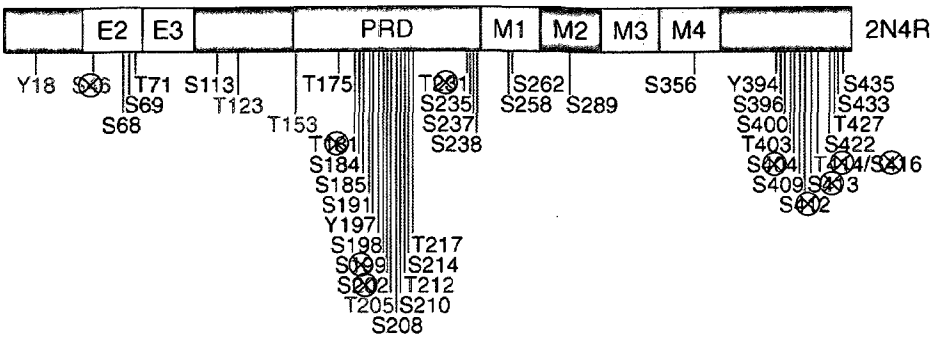



**Fig. 3.2.8. Central nervous system tau.** Six different isoforms of human tau are generated through alternative splicing of exons 2 and 3 (E2 and E3). Also indicated are proline-rich domain (PRD) and microtubule binding domains (M1-M4). 45 phosphorylation sites have been identified. Residues crossed out by blue circle are also phosphorylated in non-AD postmortem brain. Figure from Hanger et al., 2009.

Results showed that tau levels were not significantly reduced in STZ diabetic rats, and that neither IGF-I alone nor its combination with insulin altered its relative abundance (Fig. 3.2.9). This identifies tau as another protein that is largely unaffected by diabetes and which may not be under the control of either insulin or IGF.

Moreover, brain homogenates from the "12 week IGF" experiment did not exhibit an increase in phosphorylation per brain at either Ser262/Ser256 nor Ser396/Ser404 in diabetic rats. Data are reported as phospho-tau relative to total tau per brain for both antibodies (figs. 3.2.10 and 3.2.11, respectively) and there were no statistical differences between groups. This may be due to larger standard deviations within groups in this experiment. Similar data were obtained when samples from the "12 week insulin" experiment were examined (data not shown). Therefore, the STZ diabetic rats differ from AD patients and STZ mice in the pattern of phosphorylation of tau (see

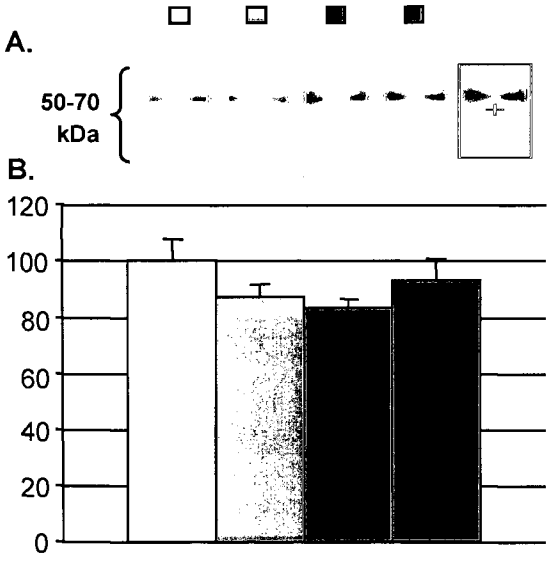



**Fig. 3.2.9. Relative total tau per brain. A.** Representative Western blot probed with anti-tau antibody. Analyzed region is shown (red box). **B.** The Non-D mean is set to 100%.

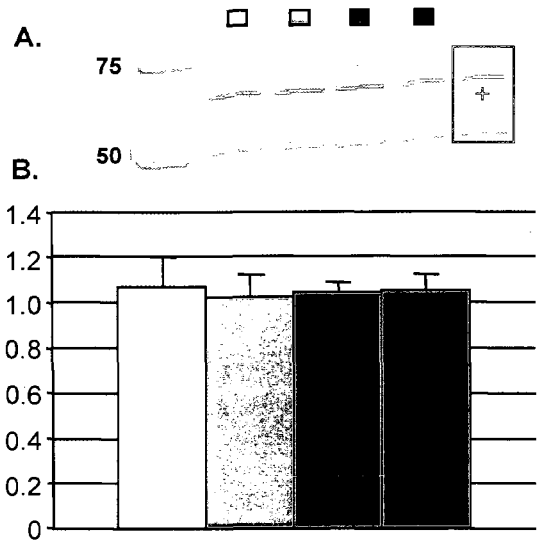



**Fig. 3.2.10. Relative pS262/pS256 tau per total tau per brain. A.** Representative Western blot probed with 12E8 anti-phospho-tau antibody. Analyzed region is shown (red box). **B.** Ratio of pS262/pS256 tau to total tau per brain.

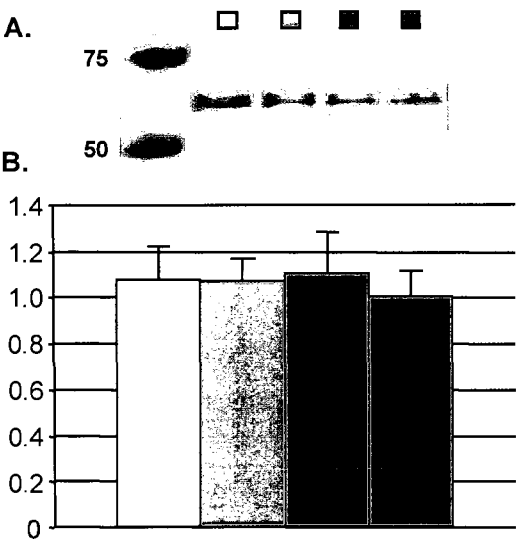



**Fig. 3.2.11. Relative pS396/pS404 tau per total tau per brain. A.** Representative Western blot probed with PHF-1 anti-phospho-tau antibody. **B.** Ratio of pS396/pS404 to total tau per brain.

Discussion). Neither reduced levels of tau nor its hyperphosphorylation, at least at the amino acid residues tested, are associated with brain atrophy in STZ diabetic rats.

## 3.2.5. EFFECT OF IGF AND ITS COMBINATION WITH INSULIN ON PLASMA AND CSF GLUCOSE

As in the "12 week insulin" experiment, tail blood was collected every 2 weeks from rats enrolled in all four groups and CSF was tapped prior to euthanasia. All diabetic animals exhibited severe systemic hyperglycemia over the entire course of the study and neither treatment with IGF-I alone nor the combination treatment affected plasma glucose levels (Fig. 3.2.12). Plasma glucose levels throughout the duration of 12 weeks resembled the pattern observed in the "12 week insulin" experiment (see Fig. 3.1.25), as did animal body weight (not shown). Likewise, CSF glucose levels were

significantly higher in all diabetic groups and neither treatment had an effect (Fig. 3.2.13).

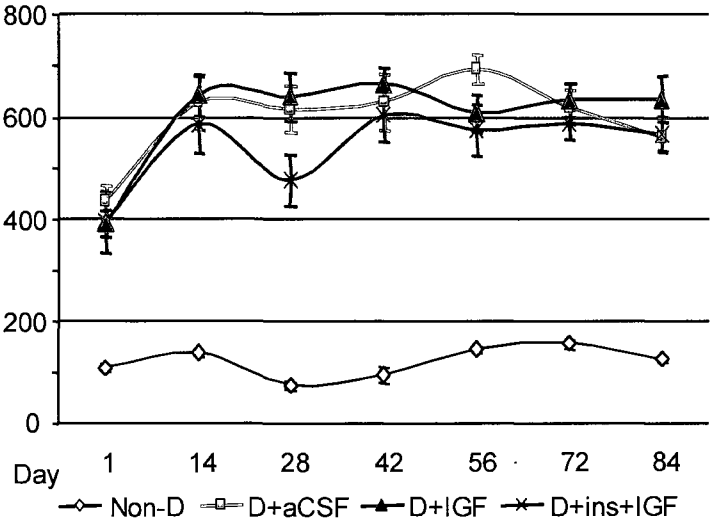



**Fig. 3.2.12.** Plasma glucose (mg/dL) throughout the "12 week IGF" experiment.

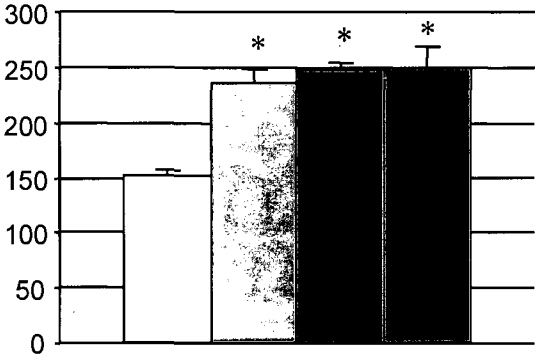



**Fig. 3.2.13.** CSF glucose (mg/dL) prior to euthanasia.

| Table 3.2.1 – Summary of statistical comparisons for the "12 week IGF" experiment | | | | |
|---|---|---|---|---|
| **Parameter** | **D+aCSF** vs. Non-D[a] | **D+aCSF** vs. D+IGF vs. D+ins+IGF[b] | **D+ins+IGF** vs. D+IGF[c] | **Non-D** vs. D+IGF vs. D+ins+IGF[d] |
| Brain wet wt. | <0.001* | <0.89<br><0.001† | <0.001‡ | <0.001*<br><0.046* |
| Brain water wt. | <0.001* | <0.87<br><0.001† | <0.001‡ | <0.001*<br><0.16 |
| Brain dry wt. | <0.008* | <0.98<br><0.001† | <0.001‡ | <0.015*<br><0.022* |
| DNA | <0.97 | <0.86<br><0.29 | <0.06 | <0.60<br><0.54 |
| Total protein | <0.001* | <0.93<br><0.001† | <0.004‡ | <0.004*<br>1 |
| GFAP | <0.001* | 1<br><0.010† | <0.010‡ | <0.001*<br><0.81 |
| NF-L | <0.001* | <0.79<br><0.05† | <0.29 | <0.005*<br><0.29 |
| NF-M | <0.002* | <0.98<br><0.018† | <0.005‡ | <0.001*<br><0.83 |

[a] Shows whether the parameter was significantly reduced in diabetes.
[b] Shows the effect of IGF-I or its combination with insulin on the parameter in diabetes.
[c] Shows whether insulin's combination with IGF-I was more effective than IGF-I alone. Notice that the combination treatment exceeded the Non-D group for brain wet and dry weights.
Significance accepted at $p<0.05$, Tukey-Kramer *posthoc* test of means.
Statistical symbols *†‡ from table correspond to symbols in figures.
For all tests in the "12 week IGF" experiment, the n-values are as follows: Non-D 9; D+aCSF 10; D+IGF 10; D+ins+IGF 10.

# PART 4. CONCLUSIONS AND DISCUSSION

## 4.1. CONCLUSIONS

Biochemical Pathology Associated With Diabetic Brain Atrophy. This study extends present understanding of the physiological and biochemical pathology associated with brain atrophy in diabetes. That pathology is characterized by decreased brain wet, water and dry weights. There was a reduction in brain content of DNA, total protein, and the abundant ubiquitous proteins actin, α-tubulin, and β-tubulin. Glia-specific proteins GFAP, PLP, and MBP were significantly reduced in the whole brain, and GFAP and PLP were qualitatively reduced in brain slices from cortex and hippocampus. Major cytoskeletal proteins NF-L, NF-M, and β-III tubulin that are associated with neurites were significantly reduced in the whole brain, and NF-M and β-III tubulin were qualitatively reduced in cortex and hippocampal slices. There was also a reduction in pre-synaptic v-SNARE proteins syntaxin and SNAP-25, as well as postsynaptic PSD-95 per brain. Levels of glutaminase and glutamine synthetase, key enzymes in the glutamine-glutamate cycle, were differently affected by diabetes. While the neuron-specific glutaminase levels were reduced, glia-localized glutamine synthetase levels were unaffected by diabetes.

Diabetes was associated with metabolic disturbances as evidenced by continuous plasma hyperglycemia throughout the 12 week study duration. Glucose levels were also elevated in the CSF and the animals on average ceased to gain weight following STZ administration. Consistent with previous observations, kidney weights were significantly greater in diabetes (not shown).

Effect of Insulin Treatment. Administration of insulin i.c.v. over the 12 week duration of the experiment completely prevented the loss of brain wet, water, and dry weights. Insulin also significantly prevented the decline of GFAP, MBP, and PLP per brain. On the other hand, it did not significantly prevent the decline in neuron-specific proteins NF-L, NF-M, β-III tubulin, syntaxin, SNAP-25, PSD-95, nor glutaminase. Insulin treatment had no effect on plasma nor CSF hyperglycemia. These data suggest that insulin alone may have stronger effects on the peripheral type insulin receptor (IR) on glial cells than on the neuronal type IR. There are indeed biochemical differences between these two receptor types (see Introduction, p. 16) but further study is needed.

Effect of IGF-I Treatment. Administration of IGF-I alone over the 12 week duration of the experiment had no effect on any parameter where there was a reduction in the case of vehicle-receiving rats. Those parameters are: brain wet, water and dry weights, brain protein content, NF-M, NF-L, and GFAP per brain. IGF-I did not have an effect on neither plasma nor CSF hyperglycemia. These data are in accordance with earlier observations that s.c. administration of IGF prevents impairments in learning and memory, but not the loss of the bulk of brain mass (Lupien et al., 2003; Lupien et al., 2006). IGF alone may have selective effects on synaptic spine density, spine plasticity, and/or on long-term potentiation.

Effect of Treatment with Insulin in Combination with IGF-I. Loss of brain wet, water, and dry weights as well as total brain protein, GFAP, NF-L, and NF-M levels were significantly prevented by the combination treatment in both experiments. In addition, insulin in combination with IGF-I prevented the decline in α-tubulin, β-tubulin, MBP, PLP, glutaminase, β-III tubulin, and syntaxin in the "12 week insulin" experiment. On the other hand, the combination treatment did not significantly prevent reductions in actin, SNAP-25, and PSD-95 per brain. Combination treatment had no effect on either plasma nor CSF hyperglycemia, nor decreased body weight and increased kidney weights.

Taken together, these data show that insulin and IGF prevented brain atrophy despite unabated hyperglycemia and reduced body weights in diabetic rats. The combination treatment was more effective than either insulin or IGF alone.

## 4.2. INFERENCES

The main inferences that can be drawn from this study are:

1. Insulin and IGF are maintenance factors for the preponderance of adult brain mass.
2. The risk of brain atrophy is increased by the concomitant decline in insulin and IGF levels in diabetes.
3. The concomitant decline in insulin and IGF levels may cause a catabolic state in brain in which there is progressive loss of RNA, protein, and ultimately cells. This may occur through reduction of synthesis of RNA and protein and/or through their increased degradation.
4. Insulin actively regulates brain water content. This is particularly important for brain edema in the clinical treatment of ketoacidosis. Brain edema may arise from inappropriately large insulin doses or too rapid administration of insulin.
5. Contrary to widespread belief, diabetes *per se* (e.g. hyperglycemia) is not the main risk factor for brain atrophy, but rather the loss of growth factor activity associated with the insulin receptor. This presents a fundamentally different hypothesis for the nature of the risk factor involved in the association of clinical diabetes with encephalopathy both in diabetes and in Alzheimer's disease.
6. Various clinical conditions in which there is a loss of both insulin and IGF activities may increase risk for brain atrophy, including obesity, diabetes, aging, and late onset Alzheimer's disease.

## 4.3. DISCUSSION

### 4.3.1. DNA LOSS IS ASSOCIATED WITH BRAIN ATROPHY IN DIABETES

Autopsy studies in clinical diabetes have shown brain lesions based on histochemical studies, but this does not reveal the magnitude of degeneration in the brain. MRI studies show atrophy, but they generally cannot distinguish between water loss alone *vs.* cellular loss. The present studies show that in STZ diabetes, brain atrophy is associated with significant DNA loss. In this section, I discuss the evidence suggesting that the extensive DNA loss in STZ diabetes is likely reflective of widespread loss of brain cells, including neurons and glia.

There is poor understanding of the magnitude of cell loss in diabetic brain encephalopathy. Loss of a few cells may be tolerated without functional consequences due to massive parallel circuits. Hence, there is need to determine the magnitude of cell loss in brain in order to assess potential impact on function. Our fluorometry measurement of DNA determines the number of cells remaining at the end of the experiment. The data show that brain DNA content is reduced by approximately 10% in diabetes. That corresponds to 10% fewer cells. Considering that adult rat brain has an estimated 330 million cells (Herculano-Houzel and Lent, 2005), a cell loss of 10% would mean that diabetic rats have lost about 33 million cells over the past 12 weeks. That magnitude is considerable, and it may at least in part account for the reduction in brain mass and learning and memory deficits observed in this model.

Even though our study is quantitative, DNA fluorometry does not distinguish between brain regions, cell types, nor the mechanism by which cells died (i.e. apoptosis, necrosis, or both). Not all cells are equally susceptible to diabetes (see p. 99). There is

supporting histochemical evidence of widespread cell death in diabetic rat brain, but the data are qualitative and do not permit evaluation of the extent of cell death. Other investigators provide histochemical data indicative of cell atrophy as well as widespread brain cell death in diabetic rats. Light microscopy reveals dark neurons and foci of neuronal loss in the hippocampal formation, dentate gyrus, amygdala, thalamus, and hypothalamus (Piotrowski, 1999). Nuclear and perikaryon diameters are reduced in hippocampal regions CA1, CA2, CA3, and the dentate gyrus (Piotrowski et al., 2001). TUNEL and cleaved caspase immunoreactivity of tissue sections show widespread loss of brain cells in agreement with our DNA findings. Cell loss is observed in the hippocampus and cortex and this may contribute to cognitive disturbances. Other brain regions also show loss of cells.

Cell Loss in Hippocampus and Cortex. Hippocampus and cortex are two brain regions frequently investigated due to their importance in learning and memory processes. (Jakobsen et al., 1987) report loss of brain mass, loss of cells, and a decrease of neocortex volume in STZ rats. There is increased TUNEL and cleaved caspase-3 immunoreactivity that is distributed uniformly and bilaterally in sensorimotor cortex and hippocampus compared to non-diabetic controls after 4-6 weeks of diabetes (Britton et al., 2003; Rizk et al., 2005; Rizk et al., 2006). Acute and chronic subcutaneous insulin treatment significantly decreases immunoreactivity for these markers of apoptosis, but this treatment also reduces hyperglycemia. Therefore, unlike in our study, one cannot rule out hyperglycemia as the main culprit for brain atrophy. A study by (Li et al., 2004) with 5-6 weeks duration of STZ-diabetes in adult rats also shows increase in TUNEL and caspase-3 immunoreactivity. This group extended their investigation to the BB/Wor rat, a genetic model of type I diabetes, where similar findings are made, although over a significantly longer duration of diabetes (8 months). In addition, there are perturbations in the ratio of the Bcl-2 protein family, shifting the

balance towards apoptosis. DNA laddering of extracted brain nucleosomal DNA is shown on agarose gels to demonstrate increased DNA fragmentation. A comparison with a simultaneously examined genetic model of type II diabetes (the BBZDR/Wor rat) shows no indication of significant increase in cell loss nor apoptotic markers despite equal levels of hyperglycemia and duration of diabetes (Li et al., 2005), suggesting that a more severe catabolic state may exist in this particular type I diabetic model. Aging is a major risk factor for clinical brain atrophy, potentially because of further reductions in insulin/IGF signaling, and whether brain atrophy is accelerated in aged diabetic rodent models is yet to be investigated.

Cell loss in Hypothalamus, Pituitary, Cerebellum, and Retina. Apoptosis is evident in the hypothalamus of adult STZ rats as early as 4 weeks of unabated diabetes by the TUNEL method (Lechuga-Sancho et al., 2006). The findings are complemented with an ELISA assay that measures cytoplasmic histone-associated DNA fragments. There is also a significant decrease in neogenesis in the dentate gyrus by 8 weeks, as determined by Western blots measuring PCNA and phosphorylated histone H3 levels. In addition to increased TUNEL and cleaved caspase-3 immunoreactivity, there is hypertrophy of microglia and reduced density of vasopressin-releasing neurons in the supraoptic nucleus of the hypothalamus after 6 months of diabetes (Klein et al., 2004). It should be noted, however, that the animals in this experiment were treated with baseline insulin to prolong survival. Anterior pituitaries from adult rats killed after 1, 4, 6, and 8 weeks of diabetes reveal cell death at 4 weeks and TUNEL labeling shows that primarily lactotrophs and to a smaller degree somatotrophs are affected, but not thyrotrophs, corticotrophs, or gonadotrophs (Arroba et al., 2003; Arroba et al., 2007). This shows that certain cell types are indeed more susceptible to diabetes than others. Bergmann glial processes in the molecular layer are apparently not affected, while GFAP-positive cells co-localize with TUNEL and cleaved caspase-3 immunoreactivity in adult STZ rat

cerebellum following 4 weeks of diabetes (Lechuga-Sancho et al., 2006). The eye is an outpocketing of the brain and STZ animals exhibit reduced thickness of the retina, with reduced number of ganglion cells. In long-term diabetic rats there's an average 8-fold increase in TUNEL-positive cells in the retina after 4 weeks (Barber et al., 1998). DNA fragmentation is present at about the same extent after 3, 6, and 12 months of diabetes.

The magnitude of cell death in brain slices has been estimated in several studies, and these are summarized in Table 4.3.1.

**Table 4.3.1.** Summary of TUNEL and cleaved caspase-3-positive immunohistochemistry from reports that performed regional quantification.

| MODEL | DURATION | REGION | TUNEL | Cleaved Caspase-3 | REFERENCE |
|---|---|---|---|---|---|
| STZ type I | 4-6 weeks | Cortex, hippocampus | 5-11 cells/730 $\mu m^2$ | 5-15 cells/730 $\mu m^2$ | Rizk et al., 2005 |
| STZ type II | 8 weeks | | 11-59 cells/730 $\mu m^2$ | 4-12 cells/730 $\mu m^2$ | |
| STZ type I | 5-6 weeks | Cortex, POA | 8-17 cells/500 $\mu m^2$ | 5-19 cells/500 $\mu m^2$ | Britton et al., 2003 |
| STZ type I* | 6 months | SON | 6% vs. 1% baseline | 25% vs. 5% baseline | Klein et al., 2004 |
| STZ type I | Up to 1 year | Retina | 9.5x @ 3 mo. | N/A | Barber et al., 1998 |
| | | | 3.8x @ 6 mo. | | |
| STZ type I | 8 weeks | Cerebellum | Present | 167 vs. 100 baseline | Lechuga-Sancho et al., 2006 |
| STZ type I | 5-6 weeks | Piriform / parietal cortex | 12% / 20% | 9% / 25% | Li et al., 2004 |
| Genetic type I | 8 months | Hippocampus | NMT 4% | 1-3% | Li et al., 2005 |
| Genetic type II | | | None | <1% | |

There are several important considerations with respect to TUNEL and caspase-3 immunohistochemistry. First, there is a limited time window in which to label dead or dying cells since cell death and debris clearance can be rapid. Therefore, this method produces but a snapshot in time that does not reveal the total magnitude of cell loss. Second, estimates of cell death in experimental treatments are generally compared to normal tissue. There is a time-dependent increase in TUNEL or cleaved caspase immunoreactivity. Given sufficient incubation time in assays, virtually all cells can become "positive", so that this method provides relative rather than absolute estimates

of cell loss. Concerns regarding false positives in TUNEL protocols have been raised by others (Conraads et al., 2009; Gobe, 2009). Third, constitutive nuclear localization of cleaved caspase-3 in a subset of glial cells in distinct regions, including Bergmann glia in the cerebellum, has recently been reported (Noyan-Ashraf et al., 2005) where caspase-3 performs a non-apoptotic role, possibly involving secondary processing of transcription factors. Fourth, TUNEL and cleaved caspase-3 immunoreactivity co-localization with GFAP has been reported (Lechuga-Sancho et al., 2006). This is considered by many to be a "point of no return" in the cell's decision-making whether to undergo programmed cell death. However, co-localization of TUNEL with caspase-3 has also been reported to be very poor (Brecht et al., 2001; Klein et al., 2004).

It is more difficult to accurately estimate the magnitude of brain cell loss from tissue sections where there is atrophy, as is the case in diabetes. For example, a 20 μm thick section of brain from diabetic rats would encompass a larger fraction of brain and possibly more cells than from non-diabetic rats due to tissue shrinkage and compression. Atrophied neurons can be difficult to distinguish from atrophied glia on H&E stained sections, and local expansion of glial populations as well as infiltration of microglia following local injury can make analysis of cell counts per area difficult to interpret. Cell counts and loss of specific cell types may vary by region in disease and only a tiny fraction of the total brain can be sampled in most studies. It would be difficult to extrapolate studies in limited tissue sections to the whole brain. Further study will be needed to more completely identify the regions and cell types most susceptible to atrophy in diabetes and responsive to insulin and IGFs.

There are several potential reasons why there was no detectable reduction in brain DNA content in the D+aCSF group in the "12 week IGF" experiment. The "12 week IGF" experiment was not performed in parallel with the "12 week insulin" experiment. Even a *de novo* repeat of the "12 week IGF" experiment did not result in

any measurable reduction in DNA in the D+aCSF group compared to the Non-D group, despite the fact that brain wet, water, and dry mass were reduced. We have largely excluded a procedural error in the fluorometry assay, in part because the reduction in DNA observed in the "12 week insulin" experiment is of comparable magnitude as in a preceding study (Lupien et al., 2006). But, STZ tends to oxidize at room temperature and over prolonged time; its potential reduction of potency cannot be ruled out. An inquiry regarding possible differences in the diet formula that was fed to our rats raised a distinct possibility that the chow used in the "12 week IGF" experiment contained a significantly greater content of phytoestrogens. Also known as dietary estrogens, they are a diverse group of naturally occurring non-steroidal plant compounds that structurally resemble estradiol and therefore have the ability to cause estrogenic effects that may be protective in brain. Finally, our hypothesis proposes that a catabolic state with decreased protein levels of sufficient duration and degree may be the precipitating factor leading to DNA loss. The condition of rats, diet, STZ strength, and other factors may influence how quickly in a particular experiment rats "fall over the edge" and suffer frank DNA loss. There was brain atrophy and loss of proteins in cells in the "12 week IGF" experiment, but this was not quite sufficient to result in detectable DNA loss. Perhaps a slightly longer duration of diabetes or larger number of rats may have made a difference.

In conclusion, our fluorometry data show that there was a significant reduction in the number of cells in the whole brain in the "12 week insulin" experiment. Immunohistochemistry (IHC) studies from others indicate that cell death is widespread in brain regions, but the magnitude of cell loss is unclear. The data suggest that the DNA loss involves substantial loss of neurons and glia in widespread regions of brain in diabetes. That possibility is discussed in greater detail below in connection with cell-type specific proteins.

### 4.3.2. THE COMBINATION TREATMENT INCREASES BRAIN DNA CONTENT IN DIABETIC RATS ABOVE NORMAL

The combination of insulin and IGF increased brain DNA content above that in Non-D rats in the "12 week insulin" experiment. One possibility is stimulation of cellular proliferation by IGF-I, since IGFs have been involved in cell proliferation in both development and adulthood (see Introduction, p. 30). There is evidence of a reduction in the number of BrdU-labeled cells in the dentate gyrus of adult rats after 10 days (Lee et al., 2005) and after 3 weeks (Stranahan et al., 2008) of STZ-induced diabetes. Absence of any proportionate difference in the expression of neuronal and glial markers suggests that differentiation of newly generated cells is not affected. Therefore, cell proliferation is reduced in the absence of IGF in diabetes. In our model, i.c.v. administration of IGF in the combination treatment may have stimulated cell proliferation, particularly in the dentate gyrus. However, the magnitude would be insufficient to account for most of the increase in DNA.

Leukocytes may migrate into the brain from periphery in response to inflammation in diabetes. One possibility is that larger numbers of leukocytes are recruited to the brain by the combination treatment. (Lechuga-Sancho et al., 2006a) found no evidence of activation of microglia in the cerebellum of diabetic rats, as determined through increase in PCNA Western blots. These findings are similar to those reported in both the hypothalamus (Klein et al., 2004) and retina (Zeng et al., 2000). However, rate of turnover of microglia is unknown and the combination treatment may retain a larger number of microglia in the brain.

Finally, astrocytes are known to proliferate in injury. While there is indication of astroglial activation in early stages of the disease (Lechuga-Sancho et al., 2006), GFAP levels begin to drop after the first week of diabetes. Indeed, in our study GFAP levels

are substantially reduced by the end of 12 weeks. There is no indication that IGFs stimulate astrocyte proliferation from precursor cells following hypophysectomy in adult brain (Aberg et al., 2006). This does not, however, exclude the possibility that the combination treatment may stimulate glial proliferation in diabetic rats.

### 4.3.3. EVIDENCE OF CELL TYPE-SPECIFIC DEGENERATION IN DIABETES

Our immunohistochemistry (IHC) data reveal that proteins that are selectively expressed in certain cell types were reduced in both hippocampus and cortex in diabetes and that treatment with insulin or its combination with IGF-I ameliorated or prevented such changes. However, the data do not distinguish whether there was cell atrophy with reduced antigen levels, cell loss, or both. These results should be interpreted together with the literature discussed above concerning DNA loss and TUNEL/caspase staining.

ASTROCYTES. Results show that GFAP levels were reduced in brain atrophy, and such loss was prevented by insulin and its combination with IGF. There is IHC evidence of protein loss and cell death involving astrocytes in cerebellum, hypothalamus, and potentially other brain regions. GFAP levels are increased in brain in the first week after induction of diabetes, followed by a decrease thereafter (Coleman et al., 2004; Lechuga-Sancho et al., 2006a). GFAP immunoreactivity co-localizes with TUNEL staining in the hypothalamus and the cerebellum after 4 weeks (Lechuga-Sancho et al., 2006), suggesting apoptosis of astrocytes. On the other hand, Coleman et al. (2004) report that although GFAP levels are decreased after 8 weeks of diabetes in cortex, hippocampus, and cerebellum, astrocyte counts are unchanged, as determined

through S-100B immunoreactivity on brain slices. One explanation is that astrocytes in some brain regions may be more susceptible to diabetes than astrocytes from other brain regions. In addition, cell counts in tissue slices may be subject to error without adequate correction for brain atrophy in diabetes as discussed above. Hypothalamic astrocytes are reduced in number due to increased apoptosis as well as decreased proliferation (Garcia-Caceres et al., 2008). Astrocytes also exhibit morphological changes, including decreased process projections. In our study, we found that there was a significant reduction in GFAP levels after 12 weeks, and this result may be consistent with both decreased GFAP expression in astrocytes as well as loss of astrocytes.

OLIGODENDROCYTES. Results showed that PLP and MBP are reduced in oligodendrocytes in diabetes, and that insulin and its combination with IGF can prevent such reduction. The IHC literature shows that in diabetes there is de-myelination and degeneration of oligodendrocytes. Levels of both protein and mRNA of PLP and MAG are selectively decreased in STZ rat brain (Kawashima et al., 2007). Electron microscopic examination of cuneate nucleus from long-duration STZ-diabetic rats reveals segmental delamination of myelin and dystrophic axonal and dendritic profiles in the neuropil (Dheen et al., 1994b).

Insulin may prevent loss of brain mass in part by regulating myelin. Lipoprotein lipase is an enzyme important in lipid biosynthesis and therefore myelin formation. Its activity is reduced in sciatic nerve in STZ rats after just 2 days of diabetes (Ferreira et al., 2002). Treatment with insulin for 4 days results in normalization of that activity. A relatively large fraction of dry brain mass is comprised of lipid, and diabetes may adversely affect lipoprotein lipase activity in brain as well. In addition, concentration of insulin as low as 0.33 nM in cultured fetal mouse brain cells stimulates the activity of cerebroside sulfotransferase, an enzyme that catalyzes the synthesis of sulfatide, a

major myelin glycolipid (Ferret-Sena et al., 1990). There is a dose-dependent activity response to insulin.

MICROGLIA. Hypertrophy of microglia in the supraoptic nucleus of the hypothalamus is observed by examination of OX-42-positive cells using immunohistochemistry on brain slices (Klein et al., 2004; Luo et al., 2002). There are fewer and shorter processes.

NEURONS AND SYNAPSES. Results showed that there was a decrease in levels of the neuron-specific proteins NF-L, NF-M, β-III tubulin, glutaminase, synapsin, SNAP-25, and PSD-95 in diabetic rats. There is considerable support in the IHC literature showing neuronal lesions in diabetes. Some neurons in PVN and SON of hypothalamus appear hyperchromatic or show a hypertrophic and vacuolated cytoplasm when stained with Nissl (Luo et al., 2002). There are shrunken diameters and condensed nuclei of neurons in hippocampi of STZ-diabetic rats after 10 weeks of diabetes, particularly in the CA1 region (Piotrowski et al., 2001). Mitochondrial swelling and dilation of the Golgi apparatus becomes evident under electron microscope examination. Synaptic endings appear devoid of vesicles (Piotrowski et al., 1999). Foci of neuronal loss and "dark neurons" can be found in the hippocampus, dentate gyrus, amygdala, thalamus, and hypothalamus (Piotrowski, 1999). *In vitro* cleaved caspase-3 assay on hippocampal homogenates shows increased activity, consistent with immunohistochemical studies.

Synapse-associated proteins synaptophysin and syntaxin are reduced in the hippocampus after 4 weeks of STZ diabetes. Neurons stained by the Golgi-Cox protocol reveal a decrease in the number of basal and apical dendrites and abnormalities in spine structure (Nitta et al., 2002). Morphological observation of pyramidal neurons of the prefrontal cortex, occipital cortex, and hippocampus by that method shows a decrease in the number of basal dendrites and reduction in spine density. The CA1 area of the

hippocampus is affected most, where the number of spines per unit area is reduced by half (Martinez-Tellez et al., 2005). Changes in neurite arborization in the hippocampus include retraction and simplification of apical dendrites, reduction of dendritic branch points, synaptic vesicle depletion, and mossy fiber morphological changes, as determined by Golgi stain procedure under light and electron microscopy after 9 days of STZ diabetes (Magarinos and McEwen, 2000). Mossy fibers form excitatory synaptic contacts with the proximal CA3 apical neurons. (Francis et al., 2008) show synaptophysin and choline acetyltransferase to be down-regulated in hippocampus and cortex in STZ mice and intranasal insulin treatment partially prevents that reduction.

Others report that using Western blots, PSD-95 and the presynaptic proteins synapsin I and synaptotagmin are significantly elevated after 8 week of diabetes (Lechuga-Sancho et al., 2006a). That may be in part due to loading equal amounts of protein onto SDS-PAGE gels. Since total protein content is reduced, differences between non-diabetic and diabetic groups can be attenuated by loading equal amounts of protein (see Materials and Methods, p. 43). Synaptic proteins may indeed be up-regulated in abundance in hippocampus in early stages of STZ-induced diabetes, as IHC analysis targeting synaptophysin and PSD-95 shows moderate increases of these proteins (Grillo et al., 2005). Nevertheless, the authors conclude that a re-organization of synapses appears to take place in the diabetic rat brain. Such increases may be an early compensatory mechanism prior to actual decline in levels, similar to GFAP. A study of synaptic proteins in the retina in STZ-diabetic rats demonstrates that protein and mRNA contents for synapsin, synaptophysin, VAMP2, SNAP-25, and PSD-95 are reduced using IHC, immunoblots, and RT-PCR (VanGuilder et al., 2008).

Impairments in the N-Methyl-D-Aspartate (NMDA) receptor have been documented in STZ rats in the same studies that determined impairments in learning and memory. Three weeks after STZ, NMDA receptors are regionally down-regulated in

layers I-III of parietal (primary somatosensory) and piriform (olfactory) cortex of rats, as found using tritium-labeled glutamate on slices (Bean et al., 2006). Immunoreactivity for NR2A subunit (Gardoni et al., 2002) and NR2B subunit of the NMDA receptor is reduced and phosphorylation of both NR2A/B subunits is also decreased in hippocampal post-synaptic densities of STZ rats (Di Luca et al., 1999), while insulin treatment normalizing glucose levels partially restores NR2B levels. Investigation of the NMDA currents in hippocampal pyramidal neurons from these rats are reduced and insulin treatment prevents these reductions as well. Further adding to the evidence that learning and memory impairments are linked to NMDA receptor-mediated abnormalities, administration of NMDA intraperitoneally into STZ rats significantly improves memory in passive avoidance and T-maze tasks (Grzeda et al., 2007). There is a specific and marked decrease in $GABA_B$ receptor density in the cortex (Martin et al., 1988), suggesting impairments in inhibitory brain circuitry as well.

Neurotransmitter levels are abnormal in diabetic rats. The turnover rate of dopamine in striatum of STZ rat brain is reduced in association with decreased ambulatory activity (Shimomura et al., 1988). Measurements of neurotransmitters serotonin and dopamine using an *in vivo* microdialysis method shows that basal levels of these neurotransmitters in the hippocampus are significantly reduced in STZ rats as well as a genetic type I diabetic model (WBN/Kob rats) (Yamato et al., 2004). In contrast, STZ-diabetes induced significant increases from basal levels of noradrenaline and neuropeptide Y in the hypothalamus, suggesting increased activity by neurons that secrete these peptides (Morris and Pavia, 2004). Conversion of norepinephrine to epinephrine is significantly increased and the alpha2 adrenergic receptor kinetics are significantly reduced in diabetes (Padayatti and Paulose, 1999). Treatment with insulin partly reverses these impairments.

## 4.3.4. ELECTROPHYSIOLOGICAL ABNORMALITIES IN LTP AND LTD IN STZ-DIABETIC RATS

Brain atrophy and degeneration in STZ-diabetic rats is associated with electrophysiological abnormalities and learning impairments. Similar to the clinical situation, central conduction velocity is reduced. Moreover, there are disturbances in long-term potentiation (LTP) and long-term depression (LTD), parameters closely linked to cognition. Electrophysiological testing consists of *ex vivo* measurements of conduction velocity and ability to trigger an action potential. LTP and LTD are activity-dependent enhancement or suppression of synaptic transmission, respectively, which constitutes a form of synaptic plasticity believed to be related to the cellular mechanisms of learning and memory.

There is progressive impairment in LTP in hippocampus from STZ-diabetic rats between 6 and 12 weeks of diabetes, while LTD is enhanced (Artola et al., 2005; Kamal et al., 1999). Impairments in evoked excitatory postsynaptic potentials in hippocampus have been associated with increases in action potential latencies and are abnormal at 8 weeks of STZ-diabetes (Ozkaya et al., 2007). Electrophysiological analysis of hippocampi from STZ rats that exhibit learning and memory impairments after 8 weeks of diabetes shows that the threshold for LTP induction is increased (Kamal et al., 2005), and by 12 weeks excitatory postsynaptic potentials are significantly reduced (Kamal et al., 2006). In CA1 region that appears to be the most-affected area of the hippocampus, LTD and LTP have similar magnitudes in diabetic and age-matched control rats, but in diabetic animals LTD is induced at more polarized and LTP more depolarized membrane potentials compared to controls. Diabetes produces a 10 mV leftward shift in the threshold for LTD induction and 10 mV rightward shift in the LTD-LTP crossover point of

the voltage-response curve for synaptic plasticity (Artola et al., 2005). These impairments are greater in aged diabetic rats, indicating that diabetes and aging additively impact the brain. Reduced LTP at the medial perforant path synapses in the dentate gyrus have also been recorded in type I diabetic mice (Stranahan et al., 2008).

### 4.3.5. COGNITIVE IMPAIRMENTS IN STZ-DIABETIC RATS

Both STZ mice and STZ rats exhibit learning and memory impairments that is associated with brain atrophy, and the cognitive disturbances progressively worsen with aging and disease duration similar to clinical diabetes.

The earliest documented learning and memory impairment in the adult STZ-diabetic rat is after 4 weeks of diabetes. Diabetic rats demonstrate impaired performance on Y maze, a test of immediate working memory (Nitta et al., 2002). After 8 and after 10 weeks of diabetes, statistically significant learning impairments are observed on the Morris water maze (Kamal et al., 2000). Insulin treatment commenced at the onset of the 10-week experiment prevents learning and memory impairments as well as LTP deficits (Biessels et al., 1998), but subcutaneous insulin administration also alters hyperglycemia. Insulin treatment commenced later during the study only partially prevents these deficits. Learning and memory impairments have been confirmed on a "can test", a novel spatial/object recognition task that does not involve the use of aversive stimuli such as the water maze (Popovic et al., 2001).

Learning and memory impairments in the water maze have been found in the BB/Wor rat, a genetic model of type I diabetes (Li et al., 2002). STZ-diabetic mice also exhibit learning and memory deficits when tested on Barnes circular maze (Jolivalt et al., 2008) and novel object preference test, a task that does not involve aversive motivation

(Stranahan et al., 2008). STZ-diabetic mice have relatively normal capacity for acquisition of *simple* new tasks, but display impairments in learning tasks such as the shuttle box and T-maze (Flood et al., 1990). Brands et al. suggest that such impairments reflect the diminished mental flexibility described in patients with type I diabetes (Brands et al., 2005).

Subcutaneous IGF-I treatment can prevent impaired learning and memory in the Morris water maze after 11 weeks of diabetes independently of ongoing hyperglycemia (Lupien et al., 2003). This supports our hypothesis.

### 4.3.6. INSULIN ACTIVELY REGULATES BRAIN WATER CONTENT

It is of considerable interest that treatment with insulin in our study prevented brain dehydration despite no change in the glucose gradient between plasma and CSF in diabetic rats. Cerebral dehydration may develop with reduced insulin signaling in aging and diabetes. The cerebral edema that may emerge during clinical treatment of diabetic ketoacidosis has been attributed by some investigators to changes in the osmotic gradient between the brain and blood (Edge et al., 2006; Glaser et al., 2004). Insulin can induce a rapid decrease in glucose concentration in blood relative to the brain. This may cause passive movement of water down its concentration gradient into the CNS and contribute to potentially dangerous edema. However, insulin prevented the loss of brain water (Fig. 3.1.2) although it had no effect on the glucose gradient between CSF and plasma in diabetic rats (Fig. 3.1.25 and 3.1.26). Insulin actively regulated water content, because it caused a flux of water from blood (relatively higher glucose and lower water concentration) into brain (relatively lower glucose and higher water concentration) against its own chemical gradient.

Rapid or high dose insulin treatment in ketoacidosis may potentially cause elevated intracranial pressure due to active regulation of brain water content. The mechanism remains speculative, but may involve regulation of ion fluxes or aquaporins rather than an osmotic gradient.

Similar to our study, (Izumi et al., 2003) report a 2.6 times lower CSF glucose concentration compared to plasma in STZ-diabetic rats. CSF-to-blood glucose ratio is normally 0.67, indicating that glucose homeostasis at the blood-brain-barrier may be affected. Indeed, blood-brain-barrier glucose transport is down-regulated in diabetes (Pardridge et al., 1990).

### 4.3.7. INSULIN AND IGF-I LIKELY ACTED THROUGH THEIR OWN COGNATE RECEPTORS

We observed prevention of brain mass loss by administering estimated replacement doses of insulin and IGF-I. By measuring human insulin levels in the CSF of our rats, we found that administered insulin was present in treated rats and at essentially physiological concentrations. Insulin administered at 0.35 U/day was shown to slightly alter hyperglycemia in STZ rats (previous Ishii lab unpublished data). We infused i.c.v. 0.3 IU/day (509 pmol/rat/day), and this lower dose did not alter hyperglycemia. In the ELISA we measured 200 mU/L CSF, or 6.8 ng/ml insulin. This concentration was within the biologically effective range for insulin on neurons. The $ED_{50}$ for induction of neurite outgrowth in cultured sympathetic neurons is 1 nM insulin or 5.8 ng/ml (Recio-Pinto et al., 1986). This CSF insulin concentration was biologically

active in diabetic rat brains based on prevention of brain atrophy, and is far too low to be expected to cross-occupy IGF receptors (LeRoith et al., 1992).

Infusing 1.3 µg/day IGF-I i.c.v. reverses impaired learning and memory in aged rats (Markowska et al., 1998). IGF-I was infused at 500 ng/day in our diabetic rats, and was effective in preventing atrophy in combination with insulin to a greater extent than insulin alone. (Harnish and Samuel, 1988) report that rats produce 3.7 µl/min (5.3 ml/day) CSF and CSF levels have been measured to contain 2-3 ng/ml IGF-I (Backstrom et al., 1984; Haselbacher and Humbel, 1982).

Receptor cross-occupancy takes place at supraphysiological concentrations and affinities for mutual receptors for insulin and IGF-I differ by a factor of at least 100 (Schulingkamp et al., 2000). If IGF-I acted through the IR or if it induced expression of insulin (discussed below), the effect of IGF-I treatment would have been indistinguishable from insulin treatment. Therefore, insulin and IGF-I acted in synergy and most likely acted through their own cognate receptors to prevent brain atrophy and degeneration.

### 4.3.8. INSULIN AND IGF-I ACTED IN SYNERGY TO PREVENT BRAIN ATROPHY AND DEGENERATION

Our data show that insulin and IGF were in combination more effective at preventing brain atrophy and biochemical abnormalities in diabetes than either peptide alone. There is substantial evidence in the literature that shows additive and redundancy effects of growth factors. Possible redundancy between insulin/IGF-I/IGF-II would offer a tremendous evolutionary advantage to vertebrates. Unlike in rats, IGF-II

activity is not heavily down-regulated during human development. In diabetes, IGF-II levels remain relatively high, buffering against a rapid and severe neuropathy in young humans.

In cell cultures, combinations of different factors promote oligodendrocyte survival *additively* (Barres et al., 1993), including insulin and IGFs. Oligodendrocytes and their precursors require continuous signaling by trophic factors to avoid programmed cell death in culture. Three classes of such trophic factors promote oligodendrocyte survival in vitro: 1) insulin and IGFs, 2) neurotrophins, particularly NT-3, and 3) ciliary-neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and interleukin 6 (IL-6). A single factor, or combinations of factors within the same class, promote only short-term survival of oligodendrocytes and their precursors, while combinations of factors from different classes promote survival additively. Long-term survival of oligodendrocytes in vitro requires at least one factor from each class, suggesting that multiple signals may be required for long-term oligodendrocyte survival *in vivo*.

Similarly, the SH-SY5Y neuroblastoma cell line does not exhibit neurite outgrowth in response to NGF in a serum-free medium unless physiological concentrations of insulin or IGF-II are present (Recio-Pinto et al., 1984). These cells lose the NGF receptors in absence of insulin or IGF and the same permissive effect is observed in PC12 cells. This shows that insulin and IGF-II act permissively for NGF to stimulate neurite outgrowth. Adult rat hippocampal progenitor cells show a dose-dependent increase in thymidine incorporation, total cell number, and number of cells entering mitosis in response to IGF-I, but pre-treatment of cells with fibroblast growth factor-2 is required for maximal proliferation. *In vivo*, growth factors such as IGFs do not exist in isolation. Hence, presence of other growth factors may further modulate the biological activity of IGFs and cellular responses.

## 4.3.9. INSULIN AND IGF MAY SUBSTITUTE FOR ONE ANOTHER

There has been substantial speculation that insulin and IGFs can substitute for one another's activities.

Insulin and IGF regulate mutual expression. Insulin can directly increase IGF-I and IGF-II mRNAs in cultured hepatocytes (Goya et al., 2001; Krishna et al., 1996; Phillips et al., 1991). Insulin can partially restore the loss of IGF-I (Goya et al., 1999; Scheiwiller et al., 1986) and IGF-I mRNA in rat liver (Boni-Schnetzler et al., 1989; Fagin et al., 1989). Message levels of IGF-II in brain and spinal cord are reduced after 2 weeks of STZ diabetes and insulin replacement therapy partially restores IGF-II mRNA levels in brain. IGF-I given subcutaneously to STZ-diabetic rats significantly prevents the loss of IGF-II transcripts in brain, showing that IGF-I regulates IGF-II expression independently of ongoing hyperglycemia (Armstrong et al., 2000). Continuous infusion of insulin can ameliorate the decline in IGF-I mRNA content in spinal cords of diabetic rats as well (Ishii et al., 1994). While IGF levels are low in poorly controlled diabetic rats, those levels are normalized in STZ animals which receive insulin (Busiguina et al., 1996). In clinical studies, levels of IGFs are partially restored through intensive insulin therapy in subjects matched for age and sex (Amiel et al., 1984; Tamborlane et al., 1981).

IR and IGF-I Receptor Exhibit Shared Properties. IR and the IGF-I receptor share ~85% conservation of the tyrosine kinase domain and interact with the same intracellular target proteins. Because ligands and receptors are very similar, there is receptor cross-occupancy at supraphysiological concentrations. Order of affinities is as follows: at the IR the rank order of affinity is insulin (<1 nM), IGF-II (10-50-fold), IGF-I (100-500-fold), whereas at IGF-I receptor the order is IGF-I (< 1 nM), IGF-II (2-10-fold), insulin (100-500-fold); IGF-II receptor binds IGF-I and IGF-II, but not insulin

(Schulingkamp et al., 2000). Indeed, infusion of IGF-I into normal rats causes hypoglycemia at very high doses that due to cross-occupancy of the IR (Jacob et al., 1989; Zapf et al., 1986). (Adamo et al., 1993) raise the possibility that activation of IR and IGF-I receptor may result in identical biological responses. Existence of hybrid IR/IGFR receptor complexes and the orphan receptor insulin-related receptor (IRR) supports that possibility (Moxham et al., 1989; Seta et al., 1993). There is extensive overlap and indeed co-expression of IR and IGF-I receptor in many brain regions, such as the granule cell layers of the olfactory bulb, hippocampal formation and cerebellar cortex (Bondy and Cheng, 2004). Both tend to express to a greater extent on neurons than on glia and the downstream intracellular signaling pathways of the receptors are virtually identical.

### 4.3.10. MECHANISM OF PREVENTION OF NEUROPATHY IS INSULIN/IGF-DEPENDENT AND HYPERGLYCEMIA-INDEPENDENT

Our data show that hyperglycemia is not the primary culprit responsible for brain atrophy and degeneration. Insulin does not gate the bulk glucose uptake in brain. fMRI shows localized glucose utilization in association with neuronal activity. If insulin were to alter glucose utilization after meals, this may disturb neuronal function which would not be desired. This may explain in part why IR in neurons may be uncoupled from glucoregulation. Indeed, others have found that LTP is not impaired in hippocampal slices from healthy rats when changing the extracellular glucose concentration over the range of 5 to 30 mmol/l, concentration that is comparable to blood glucose concentrations observed in STZ-diabetic rats (Izumi et al., 2003).

Insulin or IGF Prevent Neuropathy Despite Hyperglycemia. Deficits in insulin and IGF-dependent neurotrophic support have been proposed as key mediators of neurodegeneration in diabetes (Ishii, 1995). Despite best methods to control diabetes, neuropathy persists in 40% of patients (DCCT Research Group, 1996). Expression of both IGF-I and IGF-II is reduced in sciatic nerve in STZ-diabetic rats a week after onset of disease (Wuarin et al., 1994). IGF-I and IGF-II improve rates of peripheral nerve regeneration in STZ-induced diabetic animals and reverse diabetes-induced hyperalgesia (Zhuang et al., 1997). IGFs are reduced also in Zucker fa/fa (type II diabetic) rats and IGF-II treatment is effective in preventing hyperalgesia independently of hyperglycemia also in that model (Zhuang et al., 1997). IGF-I or IGF-II administered locally or systemically in STZ-diabetic rat protect against impairment of sensory nerve regeneration following crush despite unabated hyperglycemia (Ishii and Lupien, 1995).

Low subcutaneous insulin treatment that does not affect hyperglycemia in diabetic rats significantly normalizes diabetes-induced deficits in sensory and motor nerve conduction velocity (Huang et al., 2003). Insulin also corrects mitochondrial inner membrane potential in dorsal root ganglia despite polyol accumulation and elevated sorbitol and glucose levels. Intrathecal delivery of insulin can prevent deficits in sensory and motor nerve conduction velocity as well as axonal fiber density and length in STZ-induced diabetic rats independently of correction of hyperglycemia (Singhal et al., 1997; Toth et al., 2006).

Thus, insulin and IGFs act neuroprotectively in the peripheral nerves in diabetes despite hyperglycemia. Our findings extend the current understanding of trophic effects of insulin and IGFs to the brain. Insulin receptors in the brain in STZ-diabetes are acting by their growth regulatory path rather than glucoregulatory path (see the Hypothesis Figure, p. 37). Our results provide a paradigm for distinguishing the individual actions of these two pathways by using the blood-brain-barrier to advantage.

### 4.3.11. POSSIBLE NON-GLUCOREGULATORY MECHANISM OF INSULIN/IGF IN PREVENTING BRAIN ATROPHY

The conditional neuronal IR knockout mouse (NIRKO) demonstrates no overt phenotype (Schubert et al., 2004), raising the possibility that IGFs may be compensatory to prevent brain degeneration. Both IR and IGF-I receptor share a common intracellular signaling pathway. There are four insulin receptor substrate (IRS) molecules that facilitate the downstream signaling pathway of both IR and IGF-I receptor, with IRS-3 being restricted to adipose tissue (Bjornholm et al., 2002). Phosphorylated IRS proteins activate multiple signaling pathways, including the PI3 kinase and ERK cascades that directly regulate various physiological processes. Unlike in *Irs1* knockout mice, disruption of the *Irs2* gene results in reduced neuronal proliferation during development by 50% and reduced brain mass (Schubert et al., 2003), even though these mice reach normal body weight. There is also increased apoptosis of photoreceptor cells through development in these mice, so that most are gone by 16 months of age (Yi et al., 2005). Expression of MBP and PLP is reduced and myelination is quantitatively reduced at P10 compared to controls (Freude et al., 2008). IRS-2 is known to be the common messenger molecule shared by both IR and IGF-I receptor and may simulate concomitant reduction in signaling of both insulin and IGF-I in brain. While the *Irs2* knockout mice do develop diabetes, β-cell function was experimentally restored in order to keep them viable. Thus, *Irs2-/-* mice experience brain degeneration independently of hyperglycemia.

### 4.3.12. CLINICAL IMPLICATION OF AGING AND REDUCED INSULIN/IGF SIGNALING IN PROGRESSION TO DEMENTIA

Our findings support the hypothesis that disease progression takes place. IGF and insulin activities are known to slowly decline over lifetime in non-diabetic humans (Cohen et al., 1992; Cole and Frautschy, 2007; Hall and Sara, 1984) and animals (Arvat et al., 2000; D'Costa et al., 1993; Sonntag et al., 1999) and the levels of insulin and IGFs decline further with development of diabetes or late onset Alzheimer's disease (LOAD). Studies with aged rats suggest that the decline in IR-mediated activity in cerebellum and cortex is not due to reduced availability of the IR, but a decline in insulin-induced tyrosine phosphorylation of the IR and its downstream targets (Fernandes et al., 2001). IGFs and IGF-I receptors decline in several brain regions of aging brain, but mRNA levels are not reduced, suggesting translational impairments (Sonntag et al., 1999). In a study where 32-months-old rats were preoperatively trained in behavioral tasks and subsequently implanted with osmotic minipumps to i.c.v. infuse either IGF-I or vehicle, an improvement was observed in the place discrimination task (Markowska et al., 1998). Neurogenesis declines in brains of aged mice, but is efficiently restored after i.c.v. IGF-I administration (Lichtenwalner et al., 2001). If IGF-I production is inactivated in the liver of adult mice (LID mice), circulating IGF-I decreases by as much as 85%. In young (6-month-old) mice there is no difference between LID mice and controls in spatial learning and memory as measured using the Morris water maze test. However, in LID mice aged 15 or 18 months, acquisition of the spatial task is slower than in controls. Both working as well as reference memory are impaired.

These data show that IGF levels are critical in learning and memory as age progresses. As IGF levels decline with aging, methods of safe IGF delivery to the

central nervous system may be beneficial in preventing learning and memory impairments that come as a consequence of age.

### 4.3.13. RELEVANCE OF TAU HYPERPHOSPHORYLATION TO BRAIN DEGENERATION IN DIABETES

Increased phosphorylation of certain residues on the microtubule-binding protein tau interferes with its ability to bind and stabilize microtubules. As a consequence, tau is believed to self-associate into paired helical filaments and precipitate in the form of insoluble tangle deposits. These deposits are believed to be pathogenic in several neurodegenerative disorders collectively known as tauopathies (Hanger et al., 2009). Examples are Alzheimer's disease, progressive supranuclear palsy, and Pick's disease.

Because there is shared pathology between late onset Alzheimer's disease and brain atrophy in diabetes (see below), we investigated whether residues Ser262, Ser356 and Ser396 known to be hyperphosphorylated in postmortem Alzheimer's brain tissue are increased in phosphorylation in our STZ-diabetic rat model of brain atrophy. Monoclonal antibodies 12E8 and PHF-1 are widely used to detect hyperphosphorylation at these residues. Phosphorylation levels of tau at serine residues 262/356 and 396/404 did not differ between groups in the "12 week IGF" experiment (Fig. 3.2.8). There were also no significant differences between groups in the "12 week insulin" experiment (not shown). Total tau levels per brain, as detected using an antibody that recognizes tau independently of its phosphorylation state, were invariant between groups (Fig. 3.2.9). Thus, hyperphosphorylation of tau at these residues did not precede brain atrophy in the STZ-diabetic rat. We do not exclude the possibility that tauopathy may emerge at more advanced stages of brain degeneration or that tau may be hyperphosphorylated at other

residues relevant to AD. Others have shown increased tau phosphorylation where IR/IGF-I receptor signaling is impaired:

Clinical Type II Diabetes. Western blots targeting phospho-specific tau sites on crude extracts from postmortem frontal cerebral cortices of type II diabetic patients have shown that some residues are hyperphosphorylated compared to controls. These residues are: S202, T217, S262, S396 (Liu et al., 2009). The last 3 residues have been reported to be hyperphosphorylated in postmortem AD brain (see Fig. 3.2.8) and hyperphosphorylation at S202 is associated with neurodegeneration (Kaytor and Orr, 2002). Whether hyperphosphorylation is associated with clinical type I diabetes has not been studied, to our knowledge.

Experimental Models of Diabetes. There are studies that show increased phosphorylation of tau in adult STZ mice. Using Western blots on cortex and hippocampal homogenates, (Clodfelder-Miller et al., 2006) show an average 5-fold increase in phosphorylation at multiple tau residues: T181, S199, S202, T212, T231, S262/S356, and S396/404. Similarly, (Planel et al., 2007) show time-dependent increases in phosphorylation of tau at S202/T205, S396/S404, S199, and S422 correlate with duration of STZ-induced diabetes. Note that in both reports the investigated residues S262/S356 and S396/S404 were analyzed with the same antibodies that we used in our study. The fact that we did not observe an increase in phosphorylation may be explained by the difference in species or severity of diabetes. (Li et al., 2007) show increased phosphorylation on S396 using brain slices from type II model of diabetes, the BBZDR/Wor rat. They report immunoreactive phospho-396-tau to be localized around axons and neuropil and not bound to microtubules.

Other Models of IR/IGF-I Receptor Deficiency. Tau hyperphosphorylation is increased in the IGF-I null brain at S396 and S202 (Cheng et al., 2005), as well as the *Irs2-/-* mouse at S202 and T231 (Schubert et al., 2003). Phosphorylation of tau is

increased at T231 in the NIRKO mouse (Schubert et al., 2004), and the IR knockout mouse (Schechter et al., 2005). It should be noted, however, that the IR knockout mouse dies shortly after birth. Hyperphosphorylation at this residue is believed to be AD-specific and to precede formation of paired helical filaments, i.e. an early marker of AD progression (Hampel et al., 2003). Blockade of the IGF-I receptor at the choroid plexus results in increased phosphorylation of tau at S202/T205, S199, and S208 (Carro et al., 2006).

Tau Phosphorylation may be Regulated by IR/IGF-I Receptor Signaling. One well established role of glycogen synthase kinase 3β (GSK3β) is phosphorylation of tau. GSK-3β itself is under direct control by PKB/Akt, which inactivates GSK-3β through phosphorylation at its N-terminus (Takashima, 2006). Since Akt is downstream of IR- and IGF-I receptor-mediated signaling pathway, it is tempting to speculate that hyperphosphorylation of tau may be the consequence of reduced insulin and IGFs in brain. Phosphorylation equilibrium of any protein depends on the balance of kinases and phosphatases and protein phosphatase 2A (PP2A) is known to de-phosphorylate tau. PP2A activity is reduced in STZ mice (Clodfelder-Miller et al., 2006; Planel et al., 2007).

In summary, while there are data to show that tau phosphorylation is increased at several residues in diabetes and in models with impaired insulin and/or IGF signaling, the exact relationship of hyperphosphorylated tau to brain atrophy in diabetes has yet to be established, particularly in clinical studies where almost nothing is known of that relationship. Even in Alzheimer's disease it is not clear whether plaques and tangles are a cause or a consequence of brain atrophy and degeneration. The shared clinical pathology between diabetes and late onset Alzheimer's disease (LOAD) exhibit a shared pathology, discussed next.

## 4.3.14. THERE IS SHARED PATHOLOGY BETWEEN DIABETIC DEMENTIA AND LATE-ONSET ALZHEIMER'S DISEASE

Shared Clinical Pathology. There is considerable shared pathology between diabetic dementia and late-onset Alzheimer's disease (LOAD). The prominent risk factor for dementia in both diseases is advancing age. According to the Rotterdam Study, elderly type II diabetic patients are at a doubled risk for dementia relative to age-matched non-diabetic patients ((Ott et al., 1999); see Introduction p. 7). That risk nearly doubles every 5 years past the age of 60 and is independent of cerebrovascular disease. Other population-based longitudinal studies have also found that type II diabetic patients are at considerably higher risk for dementia, such as the Honolulu-Asia Aging Study and the Mayo Study (Leibson, 1999; Peila et al., 2002). Elderly type II diabetic patients have a 30-65% increased risk for developing AD compared to age-matched subjects (Arvanitakis et al., 2004; Biessels et al., 2004). Conversely, nearly 81% of brains confirmed as AD by neuropathology have a prior clinical history of either type II diabetes or impaired fasting glucose (Janson et al., 2004). Neuropathological autopsy and MRI studies have found that white matter hyperintensities are found in brains of Alzheimer's (Brun and Englund, 1986; Sjobeck et al., 2005), as well as diabetic patients (Dejgaard et al., 1991; Jongen et al., 2007; van Harten et al., 2007). White matter hyperintensities are lesions characterized by partial loss of myelin, axons, and oligodendroglial cells. When compared with healthy controls, patients with AD have lower CSF insulin levels, higher plasma insulin levels, and reduced insulin-mediated glucose disposal, a pattern consistent with insulin resistance observed in type II diabetes (Craft et al., 1999), leading some to hypothesize that desensitization of the neuronal IR contributes to AD pathophysiology (Hoyer, 2002). AD brains show reduced IR density and tyrosine kinase activity markers (Frolich et al., 1998). Decreased insulin-degrading

enzyme (IDE) activity is reduced in AD brain tissue (Perez et al., 2000). Mice with a homozygous deletion of the *Ide* gene show a more than 50% decrease in degradation and increased cerebral accumulation of amyloid β, together with hyperinsulinemia and glucose intolerance (Farris et al., 2003). Serum IGF-I levels are also reduced in patients with AD (Murialdo et al., 2001; Watanabe et al., 2005). Progressive stages of AD are associated with greater decline in mRNA levels of brain insulin, IGF, IR, and IGF-I receptor in postmortem frontal lobe (Rivera et al., 2005). Craft group demonstrated that intranasal insulin administration in AD patients improves verbal memory without affecting plasma insulin or glucose levels (Reger et al., 2006). Likewise, peripheral insulin administration under glucose clamp raises CSF Aβ levels in healthy older humans, suggestive of clearance from the brain (Watson et al., 2003). Memory is facilitated in both cases, and in AD patients that exhibit insulin resistance more insulin is required for memory improvement (Craft et al., 2003). Rosiglitazone, an insulin sensitizer drug and a PPARγ (peroxisome proliferators-activating receptor) agonist, improves cognitive performance in both type II diabetic and AD patients (Landreth, 2006; Risner et al., 2006; Ryan et al., 2006; Watson et al., 2005). Its basis of action has not yet been established, although in animal models rosiglitazone improves memory without an effect on amyloid burden (Pedersen et al., 2006). Despite these promising results, there is controversy regarding Aβ clearance. While clinical trials involving immunization of patients to promote clearance of the amyloid burden are largely disappointing (Bayer et al., 2005; Hock et al., 2003), a recent study did not result in learning and memory improvements despite largely successful Aβ clearance from brain (Holmes et al., 2008). Nevertheless, insulin may promote salutary outcomes in AD independently of Aβ accumulation.

There is Poor Correlation Between Plaques/Tangles and Dementia. One should consider reports where development of plaques and tangles comes with age and without

a meaningful correlation with learning and memory impairments. Such reports can be found where there was no prior bias to diagnose AD based on plaque and tangle deposits. In an unselected necropsy study in an elderly (70-103 years) sample population of 209, dementia was present in 48%, of whom 64% had features indicating probable or definite Alzheimer's disease. However, 33% of the non-demented people had equivalent densities of neocortical neuritic plaques. Similarly, some degree of neocortical neurofibrillary pathology was found in 61% of demented and 34% of non-demented individuals. This shows that there is extensive overlap of intermediate AD pathology among demented and non-demented older people despite equivalent degrees of vascular pathology (Neuropathology Group of the MRC CFAS (2001)).

A report of autopsy findings of 59 elderly, well-educated volunteers that were examined longitudinally with mental status testing for up to 8 years reveals that the brains of many subjects who did not show cognitive impairments contain abundant senile plaque and tangle deposits (Davis et al., 1999). Conversely, investigations on cognitive decline among a population of 2,616 subjects above age 75 over a follow-up period of 2.4, 6, and 9 years found considerable overlap in the AD pathologies (Braak stages) among demented and non-demented (Xuereb et al., 2000). Similar findings were reported by another group (Schmitt et al., 2000).

Thus, a growing number of clinical reports indicate that plaques and tangles may arise independently of dementia status and may be a normal consequence of aging. Indeed, low levels of Aβ are detectable in CSF from individuals showing no signs of dementia (Selkoe and Schenk, 2003). In their review, (Pearson and Peers, 2006) stipulate that formation of Aβ is a physiological process and that ablation of amyloid precursor protein (APP) that generates Aβ leads to neurodegeneration, indicating an important biological function. Aβ may be pathogenic only at sufficiently high levels in brain, but its relationship to being causative or a consequence of AD remains highly

speculative. No genetic mutation has been implicated in generation of LOAD, which comprises as many as 95% of AD cases and an imbalance of insulin and insulin-like signaling may be in large measure a contributing factor in development of LOAD. We have shown that in a model of concomitantly reduced insulin and IGF bioavailability in brain, treatment with estimated replacement doses of insulin and its combination with IGF prevent brain atrophy and degeneration despite hyperglycemia. Given the above discussed shared pathology between diabetes and LOAD, this model may be relevant in understanding the pathogenesis of LOAD. Indeed, LOAD has been coined "type III diabetes of the brain" (de la Monte et al., 2006).

## 4.4. PROPOSED FOLLOW-UP EXPERIMENTS

We have shown that a low estimated replacement dose of insulin regulates adult brain mass in the type I STZ-diabetic model of brain atrophy and degeneration. Others have shown that many different cell types in virtually all brain regions are affected by diabetes (see Section 4.3.1.). Further study should focus on differentiating which cell types (astrocytes, oligodendrocytes, neurons) are affected to what magnitude by the low insulin and IGF treatments. That can be accomplished with the same model involving simultaneously processed Non-D, D+aCSF, D+ins, D+IGF, and D+ins+IGF groups that receive treatments intracerebroventricularly to keep hyperglycemia constant. Even though no effect was observed with IGF treatment alone, previous studies have shown that IGF given subcutaneously prevents impairments in learning and memory (Lupien et al., 2003). Therefore, a test of learning and memory involving Morris Water Maze would be prudent on all groups, as well as effect of IGF treatment on the relative abundance of neuron-specific markers. A collaboration with a laboratory that extensively performs

patch clamping to measure long-term potentiation and long-term depression may be of value in order to supplement learning and memory studies.

Instead of processing the whole brain, hippocampi, cortices, and cerebelli and can be separated from one another and homogenized individually. This is a problem, because it is nearly impossible to perform a perfect dissection of these brain regions. However, such procedures are commonly performed by others, and with practice and a sufficiently high number of animals enrolled in each group, standard deviations could be kept to a minimum to see significant statistical differences in measured parameters. Homogenates can be used for fluorometric DNA assays, determination of total protein content, and Western blots for detection of pro- and anti-apoptotic markers and IR/IGF-I receptor signaling pathway molecules (PI3-kinase, Akt, GSK-3β). Alternatively, the remaining numbers of neurons and non-neurons can be determined using FACS (fluorescence-activated cell sorter) on extracted nuclei from different brain regions that are labeled with ethidium bromide and antibodies directed against NeuN, a protein believed to be specifically expressed in nuclei of neurons (Portier et al., 2006). EtBr and NeuN signals can be detected by the FACS method, and the relative numbers of nuclei can be determined. Identification of astrocyte- or oligodendrocyte-specific nuclear localization proteins would be of added benefit.

ELISA assays directed against phospho-specific IR and IGF-I receptors are available, which would reveal to what extent IR and/or IGF-I receptor phosphorylation are affected between groups. This would be an important contribution to the hypothesis, since the hypothesis rests on effects that are mediated by IR and/or IGF-I receptor activation. In addition, a subset of animals from each group can be perfused with formaldehyde and processed for confocal microscopy to study co-localization of pre- and post-synaptic markers. These data could then be interpreted together with the learning and memory test performed prior to euthanasia. Co-localization of apoptotic markers

such as TUNEL and caspase-3 with cell-type specific markers would be another important contribution. Such markers can be GFAP for astrocytes, neurofilaments for neurons, and PLP or MBP for oligodendrocytes. The effects of treatments on the established widespread apoptosis and degeneration in brain would indeed be a meaningful contribution to the field.

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