DISSERTATION

THE EFFECT OF IGF-I ADMINISTRATION ON COGNITIVE IMPAIRMENT AND BIOCHEMICAL PATHOLOGY IN A RAT MODEL OF DIABETIC DEMENTIA

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SEAN BRADLEY LUPIEN ENTITLED THE EFFECT OF IGF-I ADMINISTRATION ON COGNITIVE IMPAIRMENT AND BIOCHEMICAL PATHOLOGY IN A RAT MODEL OF DIABETIC DEMENTIA BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE EFFECT OF IGF-I ADMINISTRATION ON COGNITIVE IMPAIRMENT AND BIOCHEMICAL PATHOLOGY IN A RAT MODEL OF DIABETIC DEMENTIA

Diabetic patients have impaired learning/memory, brain atrophy, and two-fold increased risk of dementia. The cause of cognitive disturbances that progress to dementia and the biochemical pathology in diabetic brain atrophy is unknown. It has been shown that apoptosis is increased in both the hippocampus and retinal cells of streptozotocin diabetic rats. Because neurotrophic insulin-like growth factor (IGF) levels are reduced in diabetic patients and rodents, and IGF can cross the blood-central nervous system barrier (B-CNS-B), the hypotheses that systemically administered IGF can prevent cognitive disturbances and reduce brain atrophy, independently of hyperglycemia and a generalized catabolic state, were tested. Latency to escape to a hidden platform in the Morris Water Maze is widely used to test spatial memory, a hippocampus-dependent task. Adult rats were rendered diabetic with streptozotocin and implanted 4 weeks later with subcutaneous pumps that released either vehicle (D + Veh) or 20 μg/day IGF-I (D + IGF). Ten-and-a-half weeks after the onset of diabetes, the latency to escape to the hidden

platform was prolonged in (D + Veh) *vs.* nondiabetic rats ($P < 0.003$). Such prolongation was prevented in (D + IGF) *vs.* (D + Veh) rats ($P < 0.03$).

After 12 weeks of diabetes, rats were euthanized and brains were excised, weighed, and the biochemical pathology was investigated. Wet brain weights ($P < 0.001$), 18S rRNA per brain ($P < 0.002$) and poly(A)$^+$ RNA ($P < 0.04$) per brain were significantly reduced in (D+Veh) vs. nondiabetic rats, and IGF-I treatment had no effect. The (mg protein)/(wet weight brain) as well as (mg protein)/(brain) were significantly reduced in (D+Veh) vs. Nondiabetic rats ($P < 0.001$), and IGF-I treatment prevented these reductions (D+IGF-I) vs. (D+Veh) ($P < 0.03$) independently of ongoing hyperglycemia.

To examine apoptosis in neuroretinal cells, the eyes were dissected from the STZ rats and placed in 4% parafomaldehyde, sectioned and stained for the apoptotic markers, TUNEL and Phospho-Akt. In diabetic rat retina, the number of TUNEL-immunoreactive cells increased approximately 6-fold in the photoreceptor layer ($P < 0.001$) and 8-fold in the inner nuclear layer ($P < 0.001$); phospho-Akt (Thr 308) immunoreactivity increased 8-fold in the ganglion cell layer ($P < 0.001$) and 3-fold in the inner nuclear layer ($P < 0.01$). Subcutaneous IGF-I treatment significantly reduced the number of TUNEL ($P < 0.001$) and phospho-Akt immunoreactive retinal cells ($P < 0.05$) in diabetic rats approximately to the level of the non-diabetic group. Elevated TUNEL and phospho-Akt immunoreactivities were localized to distinct cell layers in the retina of diabetic rats.

The data show that IGF-I can act across the B-CNS-B to prevent loss of cognition related performance in the water maze, prevent reduced protein content and prevent increased apoptosis independently of ongoing hyperglycemia in diabetic rats. The data

are consistent with a model in which a loss of IGF activity due to diabetes may contribute to cognitive disturbances. These data also show that brain atrophy may be due in part to reduced protein, mRNA and rRNA contents.

Because IGFs are involved in synapse formation and axon elongation in peripheral nerves as well as mitosis in neuroblasts, we tested the hypothesis that brain IGF is essential for learning and memory. A cannula was implanted into the left lateral ventricle of adult rats to infuse IGF-II antibody (IGF-II Ab) or pre-immune serum. Rats were subjected to a passive avoidance test of their ability to learn and remember not to instinctively enter a dark chamber after 10 days of infusion. Rats were allowed to habituated for 2 days and given an electric shock on day. On days 4-6, the latency to enter the dark chamber tested learning and memory (avoidance of shock). The rats were given a reinforcement shock upon entry. The mean latencies for all groups on days 1-3 were not significantly different. On days 4, 5, and 6, the mean latencies of the IGF-II Ab group were significantly shorter than that of the pre-immune serum group ($P < 0.04$, 0.02, 0.004, respectively). These data show that endogenous IGF in cerebrospinal fluid regulates or supports learning and memory. Learning and memory deficiencies are observed in aging humans and rodents, and IGF-II gene expression is reduced in the brain of aged rats.

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LIST OF ABBREVIATIONS

IGF	Insulin-like Growth Factor
STZ	Streptozotocin
Diab	Diabetic
MWM	Morris Water Maze
PA	Passive Avoidance
B-CNS-B	Blood-Central Nervous System-Barrier
TU	Tracker Units
IGF-Ab	IGF Antibody
PIS	Pre-Immune Serum
TUNEL	Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling.
GCL	Ganglion Cell Layer of the retina
INL	Inner Nuclear Layer of the retina
ONL	Outer Nuclear Layer of the retina
IPL	Inner Plexiform Layer of the retina
GFAP	Glial Fibrillary Acidic Protein
cDNA	Complementary DNA

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CHAPTER 1

Background and Statement of Hypotheses

I. INTRODUCTION:

Diabetic neurological complications progress in many diabetic patients despite the best efforts to control hyperglycemia. In addition to peripheral neuropathy, cognitive impairments occur and brain atrophy is observed at later stages in life. There is a great need to develop adjuvant treatments to supplement diet, exercise, oral hypoglycemic agents and insulin that may improve both peripheral and central neurological complications in diabetes. The neurobiology of insulin-like growth factors (IGFs) has been studied in animals, and a loss of IGF activity produces neurological disorders that mimic the disturbances of diabetic neuropathy. It has been proposed that a decline in IGF neurotrophic activity may be pathogenic for diabetic neuropathy. The theory predicts and tests show that IGF levels are reduced in diabetic primates, including humans, and that IGF gene expression is reduced throughout the peripheral and central nervous system in diabetic rodents. Tests further show that replacement doses of IGFs can prevent many diabetic neurological disturbances in the peripheral and central nervous system in spite of ongoing hyperglycemia and an overall catabolic state. It has been shown that intact IGF

can cross the blood-central nervous system-barrier (B-CNS-B), thus providing a clinically practical and noninvasive method for treating CNS disorders.

It was the goal of the experiments reported here to determine whether IGF-I administration could cross the blood-central nervous system-barrier (B-CNS-B) and prevent cognitive disturbances in diabetes in spite of ongoing hyperglycemia and an overall catabolic state. We found that long-term diabetes in rats also demonstrated severe brain atrophy. The biochemical pathology of diabetic brain atrophy is not understood. We therefore examined protein, messenger RNA (mRNA) and ribosomal RNA (rRNA) content in the brain as well as apoptosis in the retina of diabetic rats and the effects of IGF-I administration. The endogenous role of IGFs in the brain were also investigated here. To study the effects of IGF-II in the brain on cognitive impairments and diabetic brain atrophy, we generated transgenic mice containing an IGF-II cDNA that was intended to overexpress IGF-II specifically in the CNS.

II. DIABETES:

An estimated 17 million people, 6.2 percent of the population, in the United States have diabetes mellitus. About 5.9 million people have not yet been diagnosed. Each year, about 1 million people age 20 or older are diagnosed with diabetes. Diabetic patients are either Type 1 or Type 2. Type 1 diabetes was previously called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. It is believed to be an autoimmune disease. In diabetes, the immune system attacks the insulin-producing beta cells in the pancreas and destroys them. The pancreas then produces little or no insulin. Someone

with type 1 diabetes needs to take insulin daily to live. Type 1 diabetes accounts for about 5 to 10 percent of diagnosed diabetes in the United States. Type 2 diabetes was previously called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes. About 90 to 95 percent of people with diabetes have type 2. This form of diabetes usually develops in adults age 40 and older and is most common in adults over age 55. About 80 percent of people with type 2 diabetes are overweight. Type 2 diabetes is often part of a metabolic syndrome that includes obesity, elevated blood pressure, and high levels of blood lipids. Unfortunately, as more children and adolescents become overweight, type 2 diabetes is becoming more common in young people. When type 2 diabetes is diagnosed, the pancreas is usually producing enough insulin, but, for unknown reasons, the body is resistant to insulin. After several years, insulin production decreases and just like Type 1 diabetes, patients become hyperglycemic.

Diabetes is widely recognized as one of the leading causes of death and disability in the United States. In 1999, about 450,000 deaths occurred among adults with diabetes. Diabetes is associated with long-term complications that affect almost every part of the body. The disease often leads to blindness, heart and blood vessel disease, strokes, kidney failure, amputations, and nerve damage. Uncontrolled diabetes can complicate pregnancy, and birth defects are more common in babies born to women with diabetes. In 2002, diabetes cost the United States $132 billion. Indirect costs, including disability payments, time lost from work, and premature death, totaled $40.2 billion; direct medical costs for diabetes care, including hospitalizations, medical care, and treatment supplies, totaled $91.8 billion.

III. DIABETIC ENCEPHALOPATHY:

A. Type 1 and type 2 diabetic patients have neurological complications that extend to the central nervous system. For reviews see references (McCall, 1992; Mooradian, 1997; Gispen and Biessels, 2000). Brain atrophy is observed by MRI (Dejgaard et al., 1991; Araki et al., 1994) and computerized tomography (Soininen et al., 1992), and structural lesions are noted at autopsy, including axonal loss and the degeneration of ganglion and cortical neurons (Reske-Nielsen et al., 1965). The early onset and high incidence of central electrophysiological disturbances suggest that encephalopathy is an accompanying complications of diabetes (Pietravalle et al., 1993; Di Luca et al., 1999). Major depression, phobias, and anorexia (Lustman et al., 1988; Popkin et al., 1988) have a higher incidence in diabetic patients than in the general population.

Of particular interest, cognitive deficits are observed in diabetes (Franceschi et al., 1984; Perlmuter et al., 1984; Helkala et al., 1995; Mooradian, 1997). Both elderly type 2 diabetic patients (Perlmuter et al., 1984; Reaven et al., 1990; Tun et al., 1990; Ryan and Geckle, 2000) and type 1 diabetic patients of long duration (Ryan et al., 1993) are at increased risk. Neuropsychological performance is neither improved by intensive insulin therapy (DCCT, 1996) nor associated with repeated episodes of hypoglycemia (Austin and Deary, 1999). Cognitive disturbances progress, resulting in a two-fold increased risk of dementia even after correcting for cerebrovascular disease (Ott et al., 1999). There is a pressing need to better understand and find treatments for the cognitive disturbances that may progress to dementia.

IV. CLINICAL DIABETIC NEUROPATHY:

Type I and type II diabetic patients are at risk for diabetic neuropathy. The major pathological feature is a dying-back axonopathy that involves both unmyelinated and myelinated axons. There is a dwindling of axon calibers, reduced axonal transport, loss of synapses, loss of axons, and, ultimately, loss of neurons. Daily wear-and-tear on the nervous system requires ability for nerve regeneration, and the poor nerve regeneration associated with diabetes may explain the loss of synapses, dying-back axonopathy and loss of neurons. There is reduced conduction velocity in about 90 % of patients, but its relationship to neuropathy remains uncertain because a significantly smaller fraction of patients progress to diabetic neuropathy. Also, axons can regenerate even when conduction is blocked by tetrodotoxin. The sensory, sympathetic and motor systems may be afflicted differently. Muscular atrophy and weakness might develop as a consequence of motor deterioration. Autonomic neuropathy includes gastroparesis with abdominal bloating and pain, loss of bladder tone that sometimes requires catheterization, abnormal cardiovascular function, silent myocardial infarct, and sudden death. Half of males have impotence. The most prevalent form of this complication is a symmetrical sensory neuropathy. With progression, there is loss of sensory function leading to the inability to do simple tasks, such as turn the pages of a book. Inability to perceive pain and touch as well as impaired proprioceptors results in an increased tendency for injuries. In conjunction with poor wound healing and increased susceptibility to gangrene, approximately 100,000 limb amputations are performed on diabetic patients each year (Bild et al., 1989; Litzelman et al., 1993). For other manifestations of clinical

neuropathy, reviews on diabetic neuropathy are available (Thomas and Tomlinson, 1993; Vinik et al., 1996).

V. INTENSIVE GLUCOSE THERAPY DOES NOT PREVENT THE PROGRESSION OF DIABETIC COMPLICATIONS IN A LARGE FRACTION OF PATIENTS:

The Diabetes Control and Complications Trial in 1993 reported that intensive insulin therapy, where multiple daily injections of insulin were administered to Type 1 diabetic patients, reduced the incidence of neuropathy by 60% (DCCT, 1996). This is a very important clinical finding. Unfortunately, neuropathy continues to progress in 40% of patients despite intensive insulin therapy, showing that there is a need for new treatments in addition to glycemic control. A treatment that could ameliorate, prevent, and/or reverse diabetic neuropathy would be a major therapeutic advance, and would help reduce the nearly $100 billion overall cost of health care for diabetic patients. A disproportionately high fraction of this amount is expended for those patients with complications such as neuropathy.

A major drawback of intensive insulin therapy is that it is associated with a 3-fold increased risk of hypoglycemia that can be life threatening and about 43% of severe hypoglycemic episodes occur during sleep. Diabetic patients with neuropathy may be more susceptible to severe hypoglycemia under intensive insulin therapy (Hoeldtke et al., 1982; Santiago et al., 1984). These patients may have impaired autonomic function and, thereby, a diminished counter-regulatory system to oppose hypoglycemia. Mortality can

be increased (Teutsch et al., 1984).

Oral monotherapies have been unable to achieve the goal of intensive therapy in type 2 diabetic patients, but a combination of insulin, oral agents, and diet can partially reduce diabetic complications (UK Prospective Diabetes Study Group, 1998). Intensive therapy reduced HbA_{1C} levels by approximately 1% below results achieved with conventional therapy, resulting in a 35% reduction in the risk of complications. Therefore, complications continue to progress in a large fraction of patients in spite of the best current methods of glucose control. The incidence of major hypoglycemic adverse events is 2.3% of patients per year. Thus, it is very important for both type 1 and type 2 diabetic patients to manage blood glucose as close to normal as possible, and therapies to supplement glycemic control to specifically treat diabetic complications are much needed.

VI. STREPTOZOTOCIN MODEL OF DIABETES:

Streptozotocin (STZ) is a molecule homologous to glucose that is specifically taken up into the insulin producing beta cells of the pancreas probably through the GLUT2 transporter. Depending on the dose and route of administration, STZ will destroy a fraction of the total number of beta cells. This provides a model for Type 1 diabetes. Insulin levels decline rapidly and stable hyperglycemia occurs within 24 to 48 hours. Although these animals are insulin deficient, they can survive without insulin supplementation for a duration that is dependent on the dose of STZ administered. We have found that 50 mg/kg of STZ administered subcutaneously is lethal to about 20% of rats up to 12 weeks.

The STZ, Type 1, diabetic rat shows neurological complications within one week (Ishii and Lupien, 1995) that continually progresses with duration of diabetes. Impaired nerve regeneration and hyperalgesia, have been shown in STZ rats (Ishii and Lupien, 1995; Zhuang et al., 1996). Cognitive impairments occur (Biessels et al., 1996). Reduced neurogenesis (Jackson-Guilford et al., 2000) and increased apoptosis (Li et al., 2002b) in the hippocampus and apoptosis of retinal cells is detected in STZ rats (Barber et al., 1998). The ZDF (fa/fa) rats provide a genetic model of type 2 diabetes. These rats become spontaneously hyperinsulinemic but insulin resistant and diabetes occurs at about 5 to 6 weeks of age. The brains of (fa/fa) rats are smaller and hyperalgesia occurs (Zhuang et al., 1997). However, nerve regeneration is not reduced which may be due to sustained levels of IGF-I gene expression in nerves that declines in STZ rats. Although the etiology of type 2 diabetes is different from type 1 diabetes, type 2 diabetes eventually show similar neurological complications as type 1 diabetes possibly correlated to a decline in insulin activity. Because STZ rats are hypoinsulinemic and hyperglycemic, the STZ rat is suitable model for studying diabetic neurological complications and therapeutic approaches towards preventing or reducing neurological complications that occur in both type 1 and type 2 diabetes.

VII. OVERVIEW OF THE IGF SYSTEM:

The IGF genes are members of the insulin gene family and are about 50% homologous to insulin. The human IGF-II gene is 30 kb and the human IGF-I gene is greater than 70 kb. Alternative exon usage, splicing, and multiple poly(A) termination

sites produces multiple IGF-I and IGF-II transcripts. Because all IGF transcripts encode the complete prepro-IGF molecule, it is believed that the multiple transcripts facilitate complex, tissue-selective regulation of gene expression. The IGF-I gene is under growth hormone control in certain tissues such as liver, but is also responsive to developmental signals, nutritional status, diabetes, ageing and neural activity. The IGF-II gene is likewise responsive to developmental signals, nutritional status, diabetes, ageing and neural activity. However, it is not responsive to growth hormone. In the adult rat, the highest levels of IGF-II gene expression are found in brain, spinal cord and peripheral nerves. It is expressed in liver in humans, but not rats due to absence of the hepatic promoter. Hence, circulating IGF-II is abundant in humans but essentially absent in adult rodents. The IGF-I gene is likewise expressed in adult brain, spinal cord, peripheral nerves, and liver but its expression is more widespread among other tissues as well. IGF-II is by far the predominant IGF in brain.

IGF-I and IGF-II are protein hormones (M_r 7.5 kDa) comprised of a single polypeptide chain held together by three intrachain disulfide bonds. IGFs are members of the insulin gene family; they are endocrine, autocrine and paracrine factors. Both IGF-I and IGF-II bind to the type 1 IGF receptor, and it is believed that most actions of IGFs are through this receptor. The type 1 receptor is a plasma membrane-bound heterodimer homologous to the insulin receptor where the extracellular alpha subunits contain the IGF binding domain, and an intracellular domain of the beta subunit is a tyrosine kinase. The type 1 receptor is present on all or virtually all neurons, and is localized to the cell body, axons and nerve terminals. In addition, IGF-II binds the type 2 receptor which is

comprised of a single polypeptide located in the plasma membrane and devoid of tyrosine kinase activity. It appears to be involved in IGF-II degradation and lysosomal targeting, and may also signal possibly through a G protein mechanism. Because their three-dimensional structures resembles that of insulin, IGFs can cross-occupy insulin receptors, but this occurs only at supraphysiological concentrations.

There are six members of an IGF binding protein (IGFBP-1 through 6) family that sequester IGFs. Circulating IGFs form a trimeric complex predominantly with IGFBP-3 and the Acid Labile Subunit. IGFs in the extracellular fluid, on the other hand, are generally in the form of dimers together with one of the IGFBPs. It is believed that tissue proteases may act on these dimers to regulate the availability of free IGFs.

Neurons have access to IGFs produced in brain, spinal cord and peripheral nerve. In addition to these autocrine/paracrine sources of IGFs, circulating endocrine sources of IGFs, primarily from liver, can provide further support for peripheral neurons. Because the signaling type 1 IGF receptor binds to both IGF-I and IGF-II, these two neurotrophic ligands form a redundant back-up system for one another. The onset of neuropathy may be slower in humans because hepatic IGF-II production provides back-up neurotrophic support to partially offset the loss of circulating IGF-I in younger diabetic patients, whereas the onset may be more rapid in adult diabetic rats due to the absence of circulating IGF-II. In diabetes there is a progressive loss of autocrine, paracrine, endocrine and redundant IGF neurotrophic support and this loss is proposed to increase the risk of neuropathy.

VIII. NEUROBIOLOGY OF IGFs:

A brief review of the neurobiology of IGFs helps to explain how IGFs are implicated in diabetic neuropathy. The strength of this model is that diabetic neurological disturbances can be rationally understood from the biochemistry and physiology of IGF action in the nervous system.

IGFs are neurotrophic factors that can support and prevent damage to neurons. The neurotrophic properties of IGF-I and IGF-II were initially discovered in the 1980s. IGF-I and II were found to induce neurite outgrowth and support survival in cultured sensory, sympathetic and human neuroblastoma cells (Recio-Pinto and Ishii, 1984; Mill et al., 1985; Recio-Pinto et al., 1986). These studies were soon extended to show that IGFs can support a wide variety of CNS as well as PNS neurons.

The cloning and sequencing of the rat IGF-I (Shimatsu and Rotwein, 1987) and IGF-II (Soares et al., 1985; Soares et al., 1986) genes permitted examination of their tissue-specific and developmental expression. The IGF-II gene is selectively expressed at the highest levels in the brain, spinal cord, and peripheral nerves among tissues of the adult rat. The IGF receptors are found on neurons as well as glial cells. The expression of the IGF-II gene is closely correlated with the development of synapses (Ishii, 1989). IGFs can increase neurite (axon and dendrite) growth in cultured neurons (Recio-Pinto and Ishii, 1984; Recio-Pinto et al., 1986), by increasing the expression of the genes that encode structural proteins of axons, such as tubulins and neurofilaments (Mill et al., 1985; Wang et al., 1992). Highly purified recombinant human IGF-I and IGF-II can increase axon growth and support survival of neurons cultured from various parts of the

central (brain and spinal cord) and peripheral nervous systems. Overexpression of IGF-I in brain of transgenic mice results in brains 55% larger than normal (Mathews et al., 1988), and mouse strains with reduced IGF-I levels have underdeveloped brains (Noguchi et al., 1983; Beck et al., 1995). IGFs appear to be able to act on all or virtually all neurons in the body.

Various data suggest that the normal role of IGFs is to help maintain the nervous system in adult mammals, including humans. IGF treatment can help repair damaged nervous systems. Administration of recombinant human IGF-I (Kanje et al., 1989) or IGF-II (Glazner et al., 1993) was found to significantly increase the rate of sensory nerve regeneration in adult rats. IGF-II administration increases motor nerve regeneration as well (Near et al., 1992). IGFs are normally produced in nerves, and IGF genes are turned on to help damaged nerves regenerate (Glazner et al., 1994). Following sciatic nerve transection in neonatal rats, IGF-II administration can prevent loss of motorneurons (Pu et al., 1999). These results demonstrate that IGF treatment can help repair damaged peripheral nerves in a mammal.

Although the central effects of IGFs are not discussed here, what has emerged is the concept that IGFs are circulating and CSF neurotrophic factors that provide general support to virtually all neurons within the PNS and CNS. Other neurotrophic factors, such as neurotrophins (NGF, BDNF, NT-3) provide additional support for select populations of neurons. Review articles may be consulted on the neurobiological actions of IGFs (Recio-Pinto and Ishii, 1988; de Pablo and de la Rosa, 1995; D'Ercole et al., 1996; Ishii and Pu, 1999). These data show the broad potential that IGFs have to treat the many

different types of cells of the central and peripheral nervous systems.

IX. LOSS OF IGF CAUSES NEUROLOGICAL DISTURBANCES THAT MIMIC THOSE OBSERVED IN DIABETES:

Blocking of IGF activity in normal, nondiabetic animals will produce neuropathy with characteristics similar to that observed in diabetes. For example, anti-IGF antibodies can block sensory and motor nerve regeneration in normal rats (Kanje et al., 1989; Near et al., 1992; Glazner et al., 1993), and nerve regeneration is impaired in diabetes. Neuron loss may occur in clinical diabetes in both the peripheral and central nervous system, and this is observed in rats treated with an anti-IGF antiserum (Pu et al., 1999) and in IGF-I null mice (Beck et al., 1995). Conduction velocity (rate at which electrical signals travel down nerves) and axonal diameters are reduced in diabetic patients, and also in IGF-I knockout mice (Rabinovsky et al., 1996; Gao et al., 1999). IGF-I administration can reverse the low motor and sensory nerve conduction velocity in these mice. These data show the loss of IGF activity is a risk factor for neuropathy independently of hyperglycemia.

IGFs can increase alpha-tubulin, beta-tubulin, 68 kDa neurofilament and 170 kDa neurofilament gene expression (Mill et al., 1985; Wang et al., 1992). Consequently, a decline in IGF gene expression in diabetes might cause diabetic biochemical disturbances including reduced neurofilament and tubulin production in nerves, and tubulins are needed for assembly of microtubules. Neurofilaments regulate axonal diameters, and axonal diameters are reduced in diabetes. The absence of adequate amounts of these

major cytoskeletal proteins may result in loss of synapses and axons. Tubulins further provide tracks on which axonal transport depends, and axonal transport is disrupted in diabetes. The metabolic need would be greatest for the longest axons, and this may underlie the length-dependent axonopathy in diabetes.

X. IGF LEVELS ARE REDUCED IN ANIMAL AND HUMAN DIABETES:

A. Humans and rhesus monkeys: IGF activity is reduced in diabetic patients. Studies using age-matched patient groups found that IGF-I activity is significantly reduced by 40-50% in type I as well as type II diabetes (Tan and Baxter, 1986; Arner et al., 1989; Ekman et al., 2000). IGFBP-1 circulating levels are elevated, and this is expected to sequester and reduce the activity of IGFs (Crosby et al., 1992). Diabetic patients with neuropathy have lower serum IGF-I levels vs. diabetic patients without neuropathy or nondiabetic patients (Migdalis et al., 1995; Guo et al., 1999). Reduced numbers of IGF-I receptors are found on red blood cells of diabetic patients (Haruta et al., 1989; Migdalis et al., 1995). Serum IGF-I levels are not correlated with glycemic control, measured as HbA(1c) levels, in type I diabetic patients treated with insulin (Ekman et al., 2000). This observation may explain why neuropathy is not better controlled in insulin-treated patients.

It is critical to recognize that there is an age-dependent run-down of IGFs in humans in the later decades of life (Hall and Sara, 1984). This may explain the age-dependence of clinical neuropathy (Pirart, 1977), and the increased incidence of neuropathy after the fourth decade of life. Thus, neurons suffer the loss of IGF

neurotrophic activity as a consequence of diabetes, and IGF levels decline further with advancing age slowly over decades.

Neuropathy may be less prevalent in type I juvenile diabetics who are adolescents or young adults, because IGF-I and IGF-II activity remain relatively high in these age groups. However, in ketotic episodes IGF-I levels transiently decline, and there is transient neuropathy (Rieu and Binoux, 1985).

Young rhesus monkeys are lean and have normal glucose tolerance. As animals age, they become obese and develop impaired glucose tolerance, but have normal or slightly elevated insulin levels. Later, they become overtly type 2 diabetic. There is a decline in IGF-I activity with every stage in the progression towards diabetes (Bodkin et al., 1991). IGF-I levels are observed to decline in the prediabetic state prior to overt hyperglycemia. Thus, IGF-I levels are not correlated with glucose levels in neither monkeys nor humans. Neuropathy is known to develop (Cornblath et al., 1989). The rhesus monkey provides important support for the theory in a close genetic cousin of humans, and shows further the relationship between age-dependent decline in IGF activity and age-dependent risk of diabetic neuropathy.

B. Type 1 diabetic rats: IGF-I gene expression is profoundly reduced in liver, adrenal glands, and spinal cords of streptozotocin diabetic rats, a model of type I diabetes (Ishii et al., 1994). IGF-II gene expression is reduced in diabetic rat brain (Wuarin et al., 1996). The significant decrease in poly(A)+ RNA content per mg brain tissue (Wuarin et al., 1996) may be related to the progressive cerebral atrophy in the brains of diabetic patients (Araki et al., 1994). IGF-I and IGF-II mRNA content is reduced in sciatic nerves

early after the induction of diabetes (Wuarin et al., 1994), most likely in Schwann cells (Pu et al., 1995). Rats may quickly develop neuropathy because of a profound loss of neurotrophic activity involving insulin, IGF-I and IGF-II.

C. Type 2 diabetic rats: The ZDF (fa/fa) rats provide a genetic model of type II diabetes. These rats are obese and become spontaneously hyperinsulinemic and diabetic at about 5-6 weeks of age. IGF-II gene expression is reduced in brain, spinal cord and peripheral nerves in adult diabetic (fa/fa) *vs.* nondiabetic (+/+) littermates (Wuarin et al., 1996; Zhuang et al., 1997). The brains of (fa/fa) rats are smaller. IGF-I gene expression is reduced in liver, but not in spinal cord or nerves. IGF-I rather than IGF-II is responsible for regulating the rate of axon elongation during nerve regeneration (Pu et al., 1999) and nerve regeneration does not appear to be impaired in type 2 diabetic rats. This is in contrast to the type 1 diabetic rat where nerve IGF-I gene expression is reduced and regeneration is impaired. These findings in rats is in accordance with clinical data showing that nerve injury is more extensive in type 1 than type 2 disease.

These data show that the IGF genes are under complex regulation, and largely independent of hyperglycemia. IGF levels are reduced in the prediabetic state prior to hyperglycemia, and reduced further as a consequence of ageing. IGF-I and IGF-II gene expression are increased in cultured hepatocytes directly in response to insulin (Phillips et al., 1991; Goya et al., 2001). Consequently, IGF gene expression may decline in diabetes partially as a consequence of the reduction in insulin activity rather than in response to hyperglycemia. Because IGF-I treatment can normalize brain IGF-II gene expression in diabetic rats independently of hyperglycemia (Armstrong et al., 2000), there may be a

cascade in which a reduction of insulin activity leads to a decline in IGF-I and a secondary decline in IGF-II. Because of complex regulation, insulin may be able only to partially restore IGF levels in diabetic patients.

XI. REPLACEMENT IGF DOSES PREVENT OR REVERSE NEUROPATHY IN DIABETIC RATS:

A. IGF-I and IGF-II Treatments Prevent The Progression of Hyperalgesia: Supersensitivity to stimuli, or hyperalgesia, is a difficult management problem in diabetic patients. Hyperalgesia is observed in diabetic animals. Mechanical compression elicits paw withdrawal when rats feel uncomfortable, and the threshold force for withdrawal is gradually reduced with the onset of hyperalgesia in STZ diabetic rats. Infusion of either IGF-I or IGF-II arrested the progression of hyperalgesia (Zhuang et al., 1996). Hyperalgesia also develops in the ZDF (fa/fa) obese and spontaneously diabetic rat. IGF administration can reverse the hyperalgesia in this model of type 2 diabetes (Zhuang et al., 1997).

B. IGF-I and IGF-II Treatments Prevent or Reverse Impaired Nerve Regeneration: There is daily wear-and-tear on the nervous system, and a need to regenerate nerve terminals. Nerve regeneration is shown to be impaired in diabetes (Longo et al., 1986; Ekstrom and Tomlinson, 1989), and this may directly contribute to the loss of synapses and the dying back of axons.

Because it is known that IGFs normally regulate nerve regeneration (Kanje et al., 1989; Near et al., 1992; Glazner et al., 1993), and that IGF gene expression is reduced in

diabetic nerves (Wuarin et al., 1994), the IGF theory (Ishii, 1995) predicts that replacement IGF therapy should prevent impaired nerve regeneration in diabetes. Systemic infusion of IGF-I or IGF-II can prevent or reverse impaired nerve regeneration (Ishii and Lupien, 1995; Zhuang et al., 1996; Zhuang et al., 1997). Single daily subcutaneous injections are effective as well. These studies show that systemically administered IGFs can act on nerve cells, and the blood-nerve barrier is not an impediment.

C. IGF-I Treatment Reverses Neuraxonal Dystrophy: Daily subcutaneous injections of IGF-I can reverse ultrastructural damage to the sympathetic nervous system. This treatment reverses by 86% the neuroaxonal dystrophy in the superior mesenteric ganglion and ileal mesenteric nerves in rats diabetic for 6 months (Schmidt et al., 1999). Neuroaxonal dystrophy is the hallmark of diabetic autonomic neuropathy, characterized by swollen preterminal axons and synapses, and shown in tissues obtained at autopsy from diabetic human subjects (Schmidt et al., 1993).

D. IGF-I Treatment Prevents Impaired Wound Healing: Wounds heal poorly in diabetic patients, often progressing to gangrene and limb amputations. The poor healing is closely associated with development of neuropathy in diabetes. This is not unexpected, because nerve injuries in themselves can produce tissue atrophy, and the health of limbs is dependent on an adequate nerve supply. IGF-I mRNA levels are reduced in the gut mucosa, and gastric lesions heal poorly in diabetic rats (Korolkiewicz et al., 2000). These investigators showed that subcutaneous administration of IGF-I normalizes wound healing in diabetic rats.

XII. IGF REPLACEMENT THERAPY PREVENTS NEUROPATHY DURING ONGOING HYPERGLYCEMIA:

Many diabetic patients are unable to maintain tight glucose control, and many even with excellent glucose control develop neuropathy. A treatment to prevent diabetic neuropathy that were effective independently of glycemic state would be welcome. Systemic as well as local infusion of IGF-I or IGF-II prevents or reverses hyperalgesia (Zhuang et al., 1996; Zhuang et al., 1997), impaired nerve regeneration (Ishii and Lupien, 1995; Zhuang et al., 1996) and, impaired wound healing (Korolkiewicz et al., 2000) independently of ongoing hyperglycemia and hypoinsulinemia in type 1 diabetic rats.

In studies reversing neuraxonal dystrophy, a higher dose of IGF-I was injected, s.c, such that glucose levels were transiently significantly reduced for 1-2 hours from 427 mg/dL to 418 mg/dL (Schmidt et al., 1999). A control group was treated with a low dose of insulin to replicate the transient IGF effect on glucose, and insulin, unlike IGF, did not reverse neuraxonal dystrophy. Consequently, neuraxonal dystrophy most likely is specifically reversed by IGF rather than the transient partial reduction in hyperglycemia.

IGF treatment reversed hyperalgesia in the ZDF(fa/fa) model of type 2 diabetes independently of hyperglycemia, hyperinsulinemia and weight gain (Zhuang et al., 1997). Therefore, IGF treatment can prevent neuropathy in type 1 or type 2 diabetes irrespective of hyperglycemia or direction of weight change. Taken together, the data show that diabetic neurological disorders as well as nephropathy can be treated independently of hyperglycemia. It is clear that extensive biochemical pathways involved in the etiology of neuropathy and nephropathy are not blocked by the consequences of hyperglycemia,

and manipulation of growth factor levels can prevent diabetic complications.

XIII. IGFs ACT ACROSS THE BLOOD-CENTRAL NERVOUS SYSTEM-BARRIER:

It is generally regarded that large molecules the size of IGFs (M_r 7.5 kDa) do not readily cross the Blood-CNS-Barrier. It would appear, therefore, that to treat the CNS with IGFs an access hole would need to be drilled through the skull, a procedure associated with significant risk of surgical mishap or infection. However, recent studies show that IGFs can cross the Blood-CNS-Barrier. It was shown that, 8 min after injection of ^{125}I-IGF-I or ^{125}I-IGF-II into the carotid artery, radioactivity was detected in brain parenchyma by autoradiography of brain slices (Reinhardt and Bondy, 1994). However, the half-life of free IGF is 5-10 min, and in 8 min approximately half of the radioactive IGF would be metabolized. Because only a few percentage of the radioactivity enters the brain, it was unclear whether the radioactivity detected in brain parenchyma represented radioactive free iodine, iodinated IGF fragments, or intact IGF. This issue was resolved by withdrawing cerebrospinal fluid after injecting ^{125}I-IGF-I into the carotid artery. The radioactivity in the cerebrospinal fluid was subjected to SDS gel electrophoresis, and some of the radioactivity was observed to migrate together with authentic IGF, showing that intact IGF molecules did cross the Blood-CNS-Barrier (Armstrong et al., 2000). Further study showed that the uptake of IGF into CSF was saturable, indicating that there was an IGF transport molecule at the Blood-CNS-Barrier, and that the uptake process did not require IGF binding to IGFBP nor to the type 1 IGF receptor (Armstrong et al., 2000;

Pulford and Ishii, 2001). Calculations showed that the circulating levels of endogenous IGF could contribute substantially to the IGF in CSF, indicating that there is communication between IGF in blood and brain.

XIV. PHARMACOKINETICS OF IGFs:

The pharmacokinetics of IGFs in man has been reviewed (Ishii and Pu, 1999). The half-life of IGF-I is 8-20 hours due to the formation of complexes with IGF binding proteins. The daily production is about 40-50 μg per kg per day, and the volume of distribution is about 0.18 L/kg. Clinically efficacious levels can be maintained for at least 16 hours by the s.c. route of administration. The pharmacokinetics of IGF-II appears similar from studies in a few patients, but additional study is needed in larger numbers of subjects. These pharmacokinetic parameters indicate that clinical trials may be conducted using a single daily subcutaneous injection of IGFs. Formulation to enhance the stability and extend the duration of IGFs would not be needed.

The daily production rate indicates that the most appropriate IGF dose would be in the range of 15-40 μg/kg/day. This is a replacement dose, and replacement doses are effective in diabetic rats. There are many successful examples of replacement therapy. For example, insulin is replacement therapy for diabetes in type I diabetic patients, glucocorticoids (cortisol, prednisone, dexamethasone) are replacement therapy for Addison's Disease, and mineralocorticoids are replacement therapy for adrenal insufficiency.

Many diabetic patients are trained in self-administration of insulin, and could easily adapt to IGF. There is a 50% failure rate within 5 years of initiating use of oral

hypoglycemic agents in Type II diabetes, and many of these patients convert to insulin use. Thus, 40% of the total diabetic population (Type I & II) self-inject insulin, and daily IGF subcutaneous administration would require a minimum of patient education.

XV. STATEMENT OF HYPOTHESIS:

Consideration of the background presented here leads to the emergence of several hypothesis: i) IGF treatment can prevent brain disturbances that contribute to impaired cognitive ability in diabetes, ii) a decline in total protein, messenger RNA and ribosomal RNA content is associated with brain atrophy in diabetes, iii) IGF-I can prevent loss of brain protein content associated with diabetic brain atrophy, iv) subcutaneous IGF-I administration can reduce apoptosis in retinal cells in diabetes, v) systemically administered IGF-I can support i, ii, iii, and iv across the B-CNS-B in diabetes, vi) IGF can preserve i, ii, iii, and iv in diabetes independently of continued hyperglycemia, and vii) brain IGF normally contributes to hippocampal-based cognitive functions.

CHAPTER 2

Effect of Subcutaneous Insulin-like Growth Factor-I Administration On Cognitive Impairment In Adult Diabetic Rats

The experiments in this chapter combined with the experiment in chapter 4 will be published in the *Journal of Neuroscience Research*, 74:512-523 (2003).

Effect of Subcutaneous Insulin-like Growth Factor-I Administration On Cognitive Impairment In Adult Diabetic Rats

Sean B. Lupien, Erik J. Bluhm, and Douglas N. Ishii

I. Abstract: Diabetic patients have impaired learning/memory, brain atrophy, and two-fold increased risk of dementia. The cause of cognitive disturbances that progress to dementia is unknown. Because neurotrophic insulin-like growth factor (IGF) levels are reduced in diabetic patients and rodents, and IGF can cross the blood-central nervous system barrier (B-CNS-B), the hypothesis that systemically administered IGF can prevent cognitive disturbances, independently of hyperglycemia and a generalized catabolic state, was tested. Latency to escape to a hidden platform in the Morris Water Maze is widely used to test spatial memory, a hippocampus-dependent task. Adult rats were rendered diabetic with streptozotocin and implanted 4 weeks later with subcutaneous pumps that released either vehicle (D + Veh) or 20 μg/day IGF-I (D + IGF). Ten-and-a-half weeks after the onset of diabetes, the latency to escape to the hidden platform was prolonged in (D + Veh) *vs.* nondiabetic rats ($P < 0.003$). Such prolongation was prevented in (D + IGF) *vs.* (D + Veh) rats ($P < 0.03$). The data show that IGF-I can act across the B-CNS-B to

prevent loss of cognition related performance in the water maze independently of ongoing hyperglycemia and reduction in brain ($P < 0.001$) and whole body weight ($P < 0.001$) in diabetic rats. The data are consistent with a model in which a loss of IGF activity due to diabetes may contribute to cognitive disturbances.

II. Introduction:

Cognitive deficits are observed in diabetes (Franceschi et al., 1984; Perlmuter et al., 1984; Helkala et al., 1995; Mooradian, 1997). Both elderly type 2 diabetic patients (Perlmuter et al., 1984; Reaven et al., 1990; Tun et al., 1990; Ryan and Geckle, 2000) and type 1 diabetic patients of long duration (Ryan et al., 1993) are at increased risk. Neuropsychological performance is neither improved by intensive insulin therapy (DCCT, 1996) nor associated with repeated episodes of hypoglycemia (Austin and Deary, 1999). Cognitive disturbances progress, resulting in a two-fold increased risk of dementia even after correcting for cerebrovascular disease (Ott et al., 1999). There is a pressing need to better understand and find treatments for the cognitive disturbances that may progress to dementia.

The streptozotocin (STZ) diabetic rat is a widely used model of type 1 diabetes with hypoinsulinemia, hyperglycemia and reduced weight. The neurological disturbances include peripheral neuropathy, structural lesions in the brain (Jakobsen et al., 1987; Lincoln et al., 1989; Tay and Wong, 1992), and reduced central conduction velocity (Carsten et al., 1989; Terada et al., 1993). Spatial learning/memory is impaired (Biessels et al., 1996), and impairment is greater in aged diabetic rats (Kamal et al., 2000).

It has been proposed that a decline in insulin-like growth factor (IGF) activity contributes to diabetic neurological disturbances (Ishii, 1995). The neurotrophic properties of IGFs have been reviewed (D'Ercole et al., 1996; Ishii and Pu, 1999). Type 1 IGF (Bohannon et al., 1988; Adem et al., 1989) and type 2 IGF (Smith et al., 1988) receptors are present in the hippocampus. Circulating IGF-I levels are reduced in types 1 and 2 diabetic patients (Tan and Baxter, 1986), and such reduction is greater in patients with neuropathy than without (Migdalis et al., 1995; Guo et al., 1999). IGF-I and IGF-II gene expression are reduced in the peripheral nerves and spinal cord, and IGF-II gene expression is reduced in the brain of STZ rats (Ishii et al., 1994; Wuarin et al., 1994; Wuarin et al., 1996; Zhuang et al., 1997). IGF-II is the predominant IGF in the adult rat brain. IGF levels may be reduced in diabetes as a consequence of loss of insulin activity rather than hyperglycemia. Circulating IGF-I levels are reduced in rhesus monkeys during impaired glucose tolerance *prior* to frank diabetes (Bodkin et al., 1991). Furthermore, insulin directly increases IGF-I gene expression in cultured hepatocytes (Krishna et al., 1996). The administration of low replacement doses of IGF-I or IGF-II prevents peripheral neuropathy in type 1 and type 2 diabetic rats independently of hyperglycemia (Ishii and Lupien, 1995; Zhuang et al., 1996; Zhuang et al., 1997; Schmidt et al., 1999). Because circulating IGF-I levels are reduced in diabetic patients, and cognitive function is poorer in human subjects with low circulating IGF-I levels (Aleman et al., 1999; Kalmijn et al., 2000), diminished IGF activity may contribute to central as well as peripheral neurological disturbances in diabetes. The possibility that diabetic cognitive disturbances may be prevented by IGF replacement therapy has not been

examined.

IGF-I can cross the B-CNS-B and a substantial fraction of IGF-I in the CNS is thought to arise from the circulation (Reinhardt and Bondy, 1994; Armstrong et al., 2000). It is consequently of interest to determine whether *systemically* administered IGF-I can preserve brain function in diabetes.

III. HYPOTHESIS: The purpose of this study was to test the following hypotheses: i) IGF treatment can prevent brain disturbances that contribute to impaired spatial learning/memory in diabetes, ii) systemically administered IGF-I can support cognitive function across the B-CNS-B in diabetes, and iii) IGF can preserve brain function in diabetes independently of continued hyperglycemia.

IV. MATERIALS AND METHODS:

A. Diabetic Rat Treatment: All work was performed in accordance with the principles set forth in the NIH Guide for the Care and Use of Laboratory Animals. Harlan Wistar rats (10 week-old males) were randomly assigned to treatment groups. STZ administered s.c. produce diabetes more consistently than by i.p. (MacSweeney et al., 1995). Rats to be rendered diabetic were fasted overnight, anesthetized under 5% isoflurane-95% oxygen for 1 min and injected *s.c.* with 50 mg/kg streptozotocin (STZ) (Sigma Chem Co.) in 10 mM sodium citrate, 0.9% NaCl, pH 4.5. The following day 0.1 ml of tail blood was collected in 10 U heparin for glucose assay. Blood was centrifuged and the plasma was assayed for glucose using Glucose Diagnostic Kit 510A (Sigma

Chem. Co.). STZ-treated rats with > 360 mg/dL glucose were enrolled in the study. Rats were housed 2 per cage under a 12-12 hr light-dark cycle. Food and water were available *ad libitum*.

Four weeks following the induction of diabetes, STZ-diabetic rats were randomly divided into two groups and implanted subcutaneously in the mid-back with osmotic minipumps (Durect Corp., Cupertino, CA) that continuously released either 20 μg/day of IGF-I (Gropep, Australia) or vehicle (10 mM acetic acid, pH 6.0). It was calculated that these particular pumps had a 19 day duration, consequently the pumps were replaced at 18 and 36 days after the initial pump implantation so that diabetic rats received a total of 7.5 weeks of continuous IGF or vehicle treatment by the end of the experiment. Rats were given 3 ml 0.9% saline s.c. after every surgical procedure to prevent dehydration, and once per week throughout the experiment. After 10.5 weeks from onset of the experiment, diabetic and nondiabetic rats were subjected to testing in the Morris water maze.

B. Morris Water Maze: Published methods were followed (Morris, 1984; Biessels et al., 1996). The Morris Water Maze consisted of a circular tank 203 cm in diameter containing 28°C water made opaque with non-toxic black paint (Fig. 2-1). Briefly, spatial learning and memory was tested using a hidden platform or "place test" where a platform 11.5 cm in diameter was submerged 1 cm below the surface in the center of one of four quadrants. Each of the four surrounding walls located 2 feet

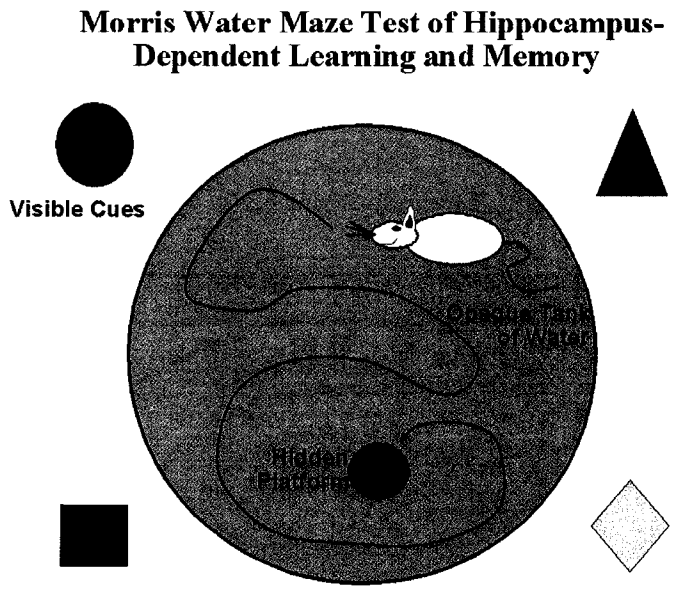

Fig. 2-1. Morris Water Maze (MWM): Hidden Platform: Spatial learning and memory was tested using a hidden platform submerged 1 cm below the surface of opaque water in one of four quadrants. Rats were randomly placed in one of the quadrants and allowed 120 sec to locate the platform using different colored symbols on the surrounding walls that served as visual cues. Rats were permitted to remain on the platform for 30 sec to permit spatial orientation. Rats were subjected to 3 daily trials, 60 sec apart, for 5 consecutive days. A video camera was mounted above the tank and swim paths were analyzed with a Poly-Track Video Tracking System (San Diego Instruments). Latency to find the platform, distance swam and swim velocity were computed. Visible Platform: Three days following the hidden platform test, a visible platform was used to detect sensorimotor deficits that may be present in diabetic rats. The platform was decorated and emerged 0.5 cm above the water. Rats were randomly placed in one of the four quadrants and allowed to swim to the platform. All rats located the platform within the allotted 120 sec. The test consisted of 6 trials spaced at 60 sec intervals, all in a single day. The platform was moved to one of six different predetermined locations for each trial.

beyond the edge of the tank had different shaped symbols that served as cues to locate the submerged invisible platform. Rats were randomly placed in one of the quadrants against the side of the tank and allowed up to 120 sec swimming time to locate the platform. Rats that had located the platform, or had failed and were placed on the platform, were permitted to remain there for 30 sec to permit spatial orientation. Thereafter, rats were removed and placed under a heat lamp between trials. Rats were subjected to 3 daily trials, with a 60 sec interval between trials, for 5 consecutive days. A video camera was mounted above the tank and swim paths were analyzed with a Poly-Track Video Tracking System (San Diego Instruments). Latency to find the hidden platform, distance swam and swim velocity were computed. Distance is reported in tracker units (TU), where 1 TU=1.1 cm.

Three days following the hidden platform, a "probe" test was used to examine memory. The platform was removed from the tank. Rats were placed in the tank and the number of seconds spent in a defined area (radius = 45 cm; centered at the former position of the hidden platform) was measured following a single swim. The swim time was limited to 120 sec.

Immediately after the probe test, a visible platform or "cued test" was used to detect sensorimotor deficits that may be present in diabetic rats. The platform emerged 0.5 cm above the water and was decorated with an overhanging yellow ball and red cone. Rats were randomly placed in one of the four quadrants and allowed to swim for up to 120 sec to locate the platform. All rats located the platform within 120 sec. The test consisted of 6 trials spaced at 60 sec intervals, all in a single day. The platform was

moved to one of six different predetermined locations for each trial.

One day after the visible platform test, rats were weighed, tail blood was withdrawn for glucose assays, and rats were euthanized with excess carbon dioxide. Various tissues were excised for determination of brain and hippocampus weights and other future measurements.

C. Statistical Analysis: The water maze data were analyzed with a computer program, CSS: Statistica (StatSoft, Tulsa, OK), using ANOVA with repeated measures. Interaction effects were analyzed further by contrast analysis. The values shown are means $\pm$ SEM.

V. RESULTS:

A. Effect of Subcutaneous IGF-I Administration on Performance of Diabetic Rats in a Morris Water Maze Hidden Platform Test: In order to test whether systemically administered IGF-I could prevent cognitive disturbances in diabetes, adult rats were randomly assigned to one of three treatment groups (see Methods). After 10.5 weeks of diabetes, rats were subjected to the Morris water maze hidden platform (place) test. Rats instinctively normally want to "escape" the water and are highly motivated to locate the platform.

1. Latency to Escape to a Hidden Platform: There was a significant decrease in the latencies to escape to the hidden platform over the 5 days of the test in all 3 groups: (Non-Diabetic), $P < 0.001$; (Diabetic + Vehicle), $P < 0.023$; (Diabetic + IGF-I), $P < 0.014$ (Fig. 1). This showed that all rats were at least capable of detecting visual cues,

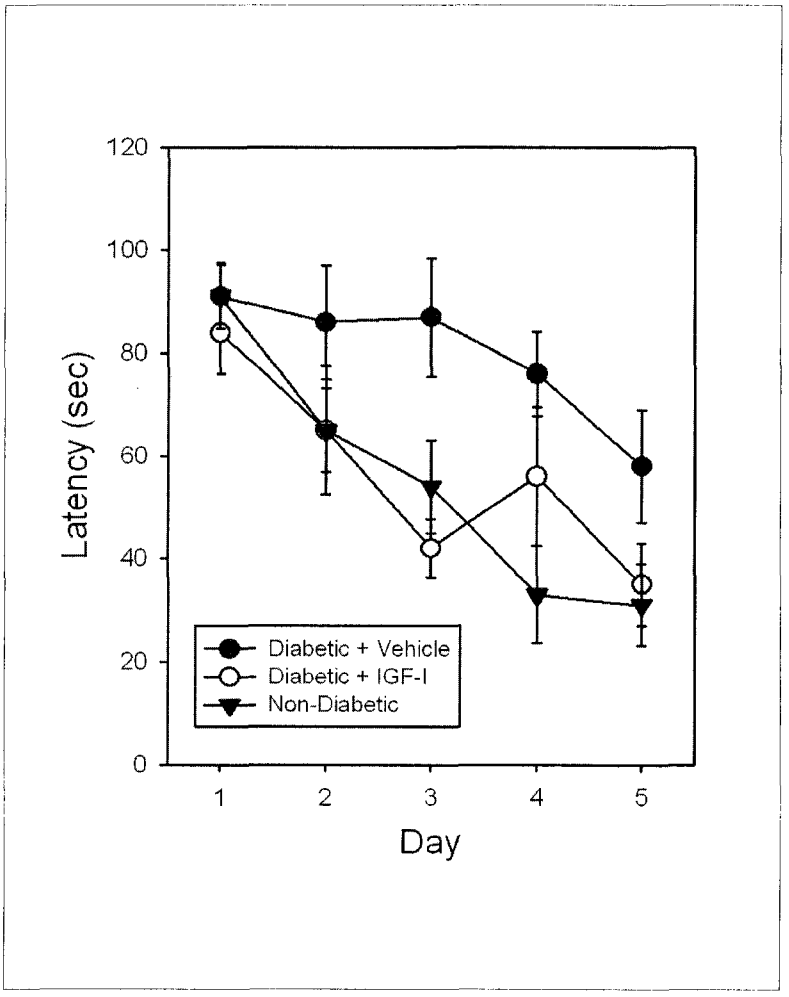

Fig. 2-2. Subcutaneous IGF-I administration in diabetic rats prevented the development of prolonged latency to escape to a hidden platform in the Morris water maze. Rats were rendered diabetic by STZ for 12 weeks and then implanted subcutaneously with osmotic minipumps that released either 20 μg/day IGF-I (Diabetic + IGF-I) or vehicle (Diabetic + Vehicle) for the final 8 weeks. After 10.5 weeks of diabetes, rats were subjected to 5 days of trials in the Morris water maze hidden platform test. The latency to escape to the hidden platform was significantly decreased between Days 1 and 5 in all 3 groups as shown by the negative slopes (Diabetic + Vehicle), $P < 0.023$, $N = 9$ rats; (Diabetic + IGF-I), $P < 0.014$, $N = 7$; (Non-Diabetic), $P < 0.001$, $N = 12$. The mean latency (average over the 5 days) was significantly increased in the (Diabetic + Vehicle) *vs.* (Non-Diabetic) rats ($P < 0.003$). The mean latency was significantly reduced in (Diabetic + IGF-I) *vs.* (Diabetic + Vehicle) rats ($P < 0.03$), but was *not* different between (Diabetic + IGF-I) *vs.* (Non-Diabetic) rats. The data were analyzed by ANOVA with repeated measures. The symbols are means $\pm$ SEM.

which is required to improve performance with repeated trials. The time course for the decrease in latency was the same in the (Non-Diabetic) and (Diabetic + IGF-I) groups; there was an immediate, progressive decline in latencies throughout the 5 days of testing. By contrast, there was a 3 day lag before latencies began to decline in the (Diabetic + Vehicle) group. This lag was eliminated in the (Diabetic + IGF-I) group.

The mean latency (5-day average) was significantly prolonged in (Diabetic + Vehicle) *vs.* (Non-Diabetic) rats ($P < 0.003$), showing poorer performance by the vehicle-treated rats that had been diabetic for 10.5 weeks. This poorer performance was almost completely prevented when IGF-I was subcutaneously infused during the final 7.5 weeks of diabetes. There was a significant reduction in the mean latency in (Diabetes + IGF-I) *vs.* (Diabetes + Vehicle) rats ($P < 0.03$).

2. Distance Swam to Escape to a Hidden Platform: A reduced latency to escape to the hidden platform might result from reduced distance swam, increased velocity of swimming, or both. The distance swam significantly decreased over the 5 day test in the (Non-Diabetic)($P < 0.001$) and (Diabetic + IGF-I) ($P < 0.027$) groups (Fig. 2-3), showing shortening of their search path for escaping to the hidden platform was the cause of their reduced latencies. By contrast, the distance swam by the (Diabetic + Vehicle) group curiously did not significantly change over the 5 days ($P < 0.292$), suggesting that they were instead increasing swim velocity.

The forgoing results were further reinforced by analysis of the rate at which the distance swam was reduced over the 5 days of testing (negative slope). There was a significant difference in slopes between (Non-Diabetic) *vs.* (Diabetic + Vehicle) rats

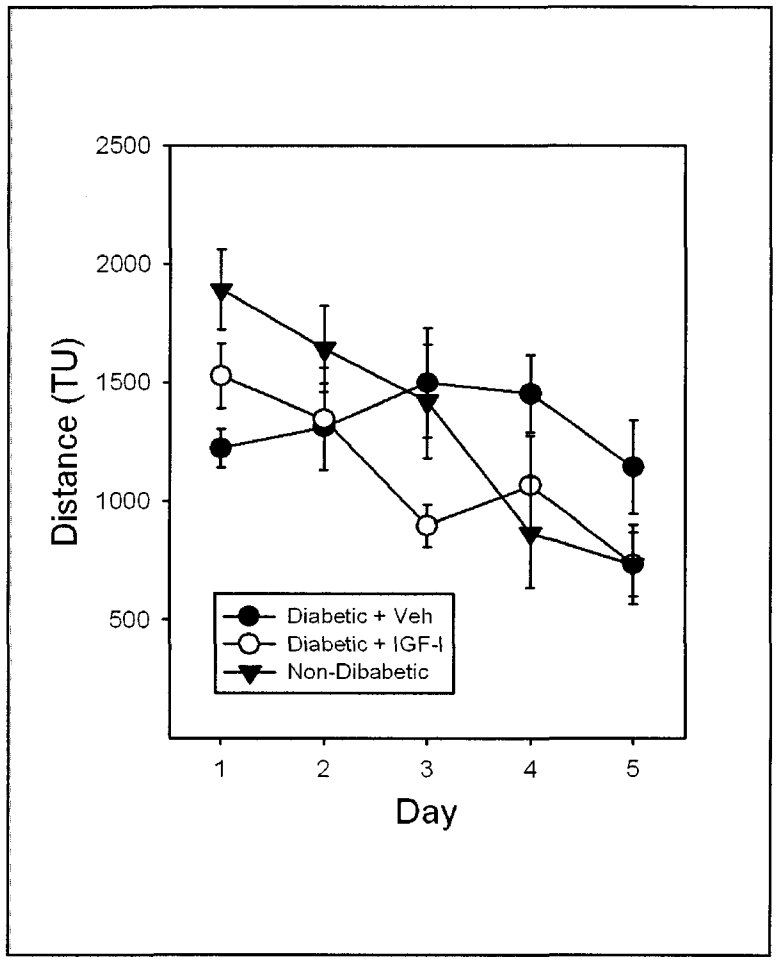

Fig. 2-3. The distance swum to escape to a hidden platform was reduced in nondiabetic but not diabetic rats, and IGF-I administration prevented this abnormality in diabetic rats. There was a progressive reduction over 5 days in the distance swum to escape to a hidden platform (negative slope) in (Non-Diabetic) ($P < 0.001$) and (Diabetic + IGF-I) ($P < 0.03$) rats, but not in the (Diabetic + Vehicle) group ($P = 0.3$). Furthermore, there was a significant difference between the slopes of the (Diabetic + Vehicle) vs. (Non-Diabetic) groups ($P < 0.003$) as well as (Diabetic + IGF-I) vs. (Diabetic + Vehicle) groups ($P < 0.03$). Distances are shown in trial units (1.1 cm/unit). The data were analyzed by ANOVA with repeated measures to test for significant interactions (differences) between slopes. The data were analyzed by ANOVA with repeated measures. The symbols are means $\pm$ SEM.

($P < 0.003$). Likewise, there was a significant difference in slopes between (Diabetic + IGF-I) vs. (Diabetic + Vehicle) rats ($P < 0.03$). By contrast, there was no difference in slopes between (Non-diabetic) and (Diabetic + IGF-I), showing that these groups more rapidly reduced the distance swam to escape to the hidden platform than did (Diabetic + Vehicle) rats.

3. Swim Velocity to Escape to a Hidden Platform: After the first day, the swim velocity appeared to slightly increase in the (Nondiabetic) and (Diabetic + IGF-I) groups, and was constant thereafter (Fig. 2-4). By contrast, there was a slow and significant increase in swim velocity in the (Diabetic + Vehicle) group from 14 TU/sec on Day 1 to 21 TU/sec on Day 5 ($P < 0.001$).

B. Effect of Subcutaneous IGF-I Administration on Performance in A Probe Test: Three days following the place testing, the hidden platform was removed altogether and the capacity of rats to remember the position of the previously hidden platform was tested. This was done by measuring the time rats spent swimming in an area with radius of 45 cm, centered at the location of the removed platform.

There were no significant differences between group means. The mean $\pm$ SEM duration spent swimming in the defined area for each group were: (Nondiabetic), 28.5 $\pm$ 2.0 sec; (Diabetic + Veh), 23.3 $\pm$ 3.5 sec; (Diabetic + IGF-I), 28.1 $\pm$ 3.7 sec.

C. Effect of Subcutaneous IGF-I Administration on Performance of Diabetic Rats in A Morris Water Maze Visible Platform (Cue) Test: The water maze parameters can be modified to test for sensorimotor impairments. Three days following the last day of place testing and immediately following the probe test, rats were subjected

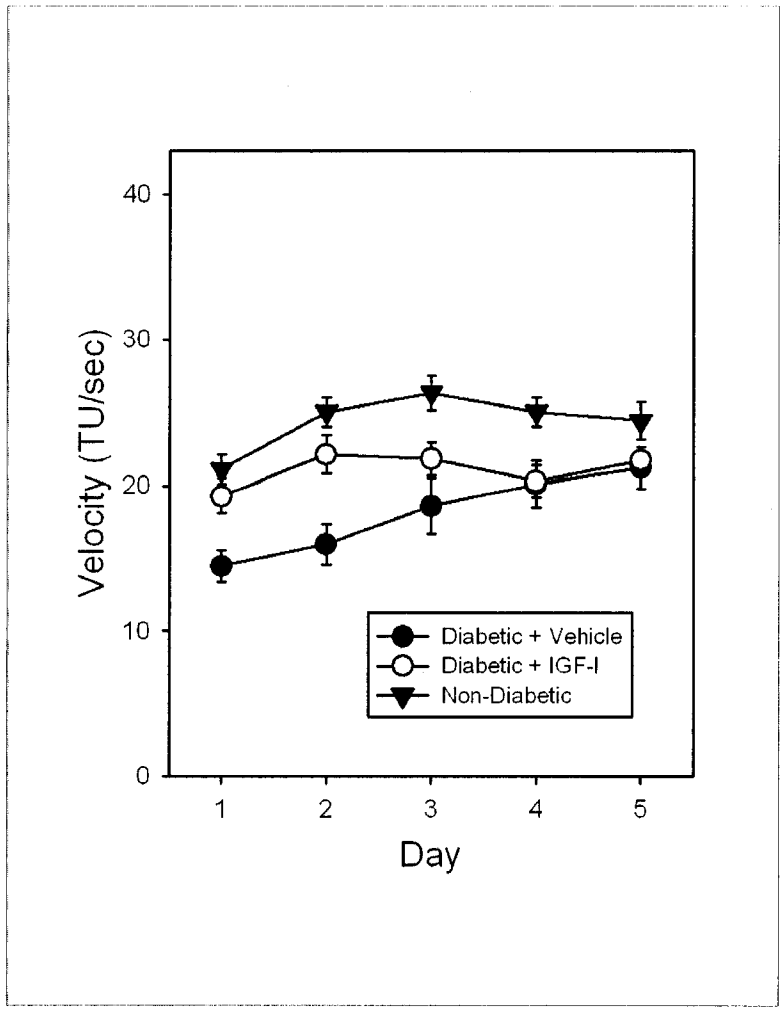

Fig. 2-4. Diabetic rats initially swam more slowly than non-diabetic rats in the hidden platform test but eventually swam with the same velocity as non-diabetic rats. There appeared to be a small increase in velocity after Day 1, but there was no change thereafter in (Non-Diabetic) and (Diabetic + IGF-I) groups. The (Diabetic + Vehicle) rats initially swam more slowly than the other groups, then progressively swam more rapidly throughout the 5 test days (positive slope) ($P < 0.001$). Using contrast analysis, there were no significant differences in velocity between any of the groups on the last day of the test. The slope for change in velocity over the 5 test days was significantly greater in (Diabetic + Vehicle) *vs.* (Non-Diabetic) rats ($P < 0.01$). IGF-I treatment prevented this abnormality, as shown by the significant difference in slope in (Diabetic + IGF-I) *vs.* (Diabetic + Vehicle) rats ($P < 0.007$). There was no difference in slopes between (Diabetic + IGF-I) vs. (Non-Diabetic) rats, showing normalization. Velocities are shown in trial units (1.1 cm/unit) per sec. The data were analyzed by ANOVA with repeated measures. The symbols are means $\pm$ SEM.

to a test of their ability to escape to a visible platform whose position was moved to a different location for each trial. Six trials were conducted in a single day.

1. Latency to Escape to a Visible Platform: The latency was sharply reduced from the last day of the hidden platform test to the first day of the visible platform test for all groups, showing that rats were being guided most likely by visual cues. The latency to escape to the visible platform did not significantly change over the 6 trials for all three groups (Fig. 2-5). This showed that learning was not a factor. However, the mean latency (average over the six trials) was significantly prolonged in (Diabetic + Vehicle) vs. (Non-Diabetic) rats ($P < 0.015$). This prolongation was prevented in the (Diabetic + IGF-I) rats. IGF-I treatment reduced the latency by 70 % in diabetic rats.

2. Distance Swam to Escape to a Visible Platform: The distance swam to escape to the visible platform did not significantly change over the 6 trials for all three rat groups (Fig. 2-6). However, the mean distance swam (average of the six trials) was significantly further in (Diabetic + Vehicle) vs. (Non-Diabetic) rats ($P < 0.015$). On the other hand, the mean distance swam was *not* significantly further in the (Diabetic + IGF-I) rats, showing that IGF-I treatment had prevented this disturbance. IGF-I treatment reduced the mean distance swam by 58% in diabetic rats.

3. Swim Velocity to Escape to a Visible Platform: The mean swim velocities were not significantly different in any of the groups (Fig. 2-7). Furthermore, there was no change in velocity over the six trials. The diabetic and nondiabetic rats were all capable of swimming with the same velocity, showing that diabetes did *not* cause

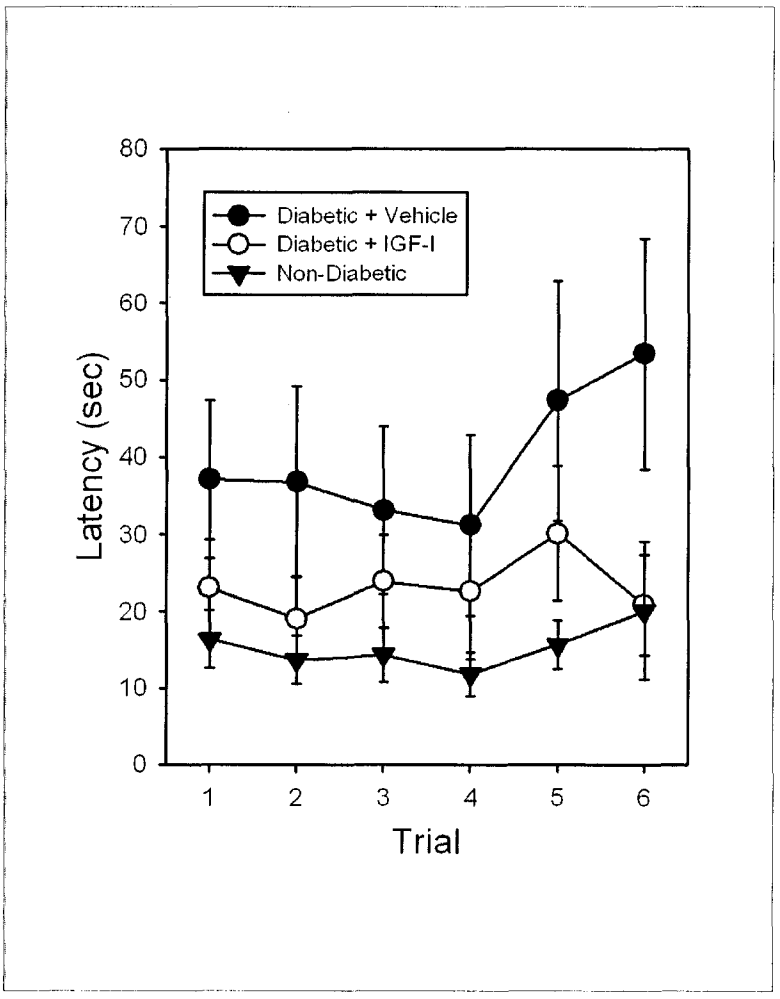

Fig 2-5. The latency to escape to a visible platform was longer in diabetic than nondiabetic rats, and this abnormality was prevented by IGF-I treatment in diabetic rats. Three days after completion of the hidden platform test, the visible platform (cued) test was conducted consisting of six trials in a single day. The latency to escape to a visible platform did not significantly change over the 6 trials for any group. The mean latency (average over the six trials) to reach the visible platform was significantly longer for the (Diabetic + Vehicle) *vs.* (Non-Diabetic) rats ($P < 0.015$). Treatment with IGF-I eliminated this significant difference: (Diabetic + IGF-I) *vs.* (Non-Diabetic), $P < 0.08$. The data were analyzed by ANOVA with repeated measures. The symbols are means $\pm$ SEM.

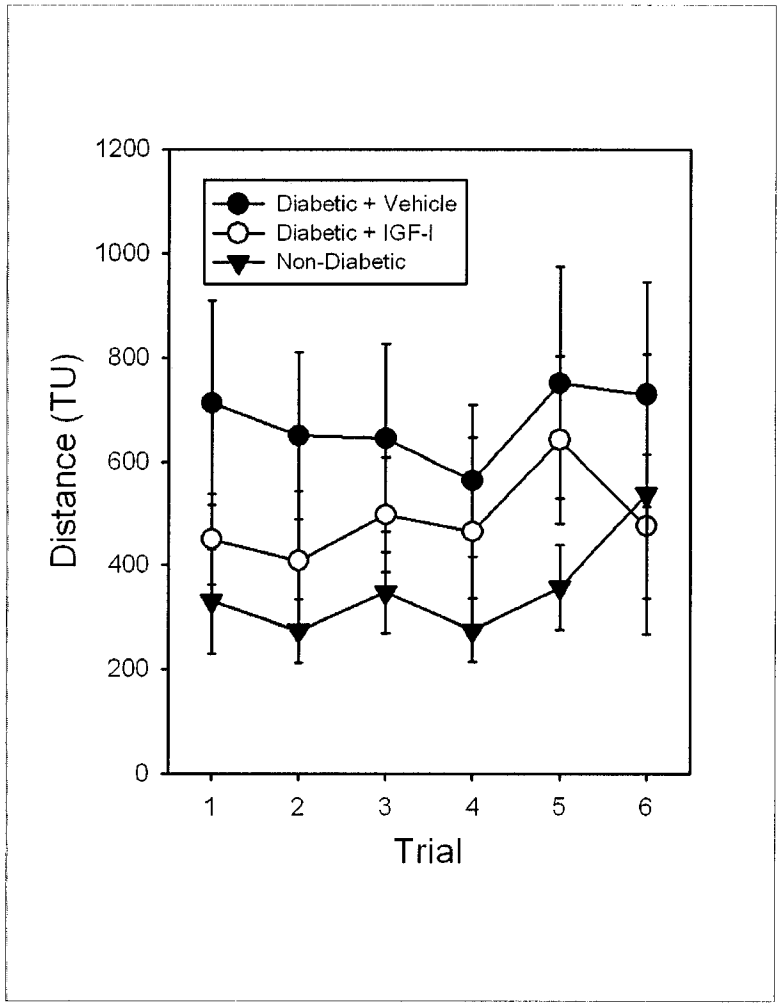

Fig. 2-6. The distance swum to escape to a visible platform was greater in diabetic than nondiabetic rats, and this abnormality was prevented by IGF-I administration in diabetic rats. The distance swum to the visible platform did not significantly change over the 6 trials for any group. The mean distance (average over the six trials) swum was significantly further by the (Diabetic + Vehicle) *vs.* (Non-Diabetic) Group ($P < 0.015$). Treatment with IGF-I eliminated this significant difference: (Diabetic + IGF-I) *vs.* (Non-Diabetic), $P < 0.2$. The data were analyzed by ANOVA with repeated measures. The symbols are means $\pm$ SEM.

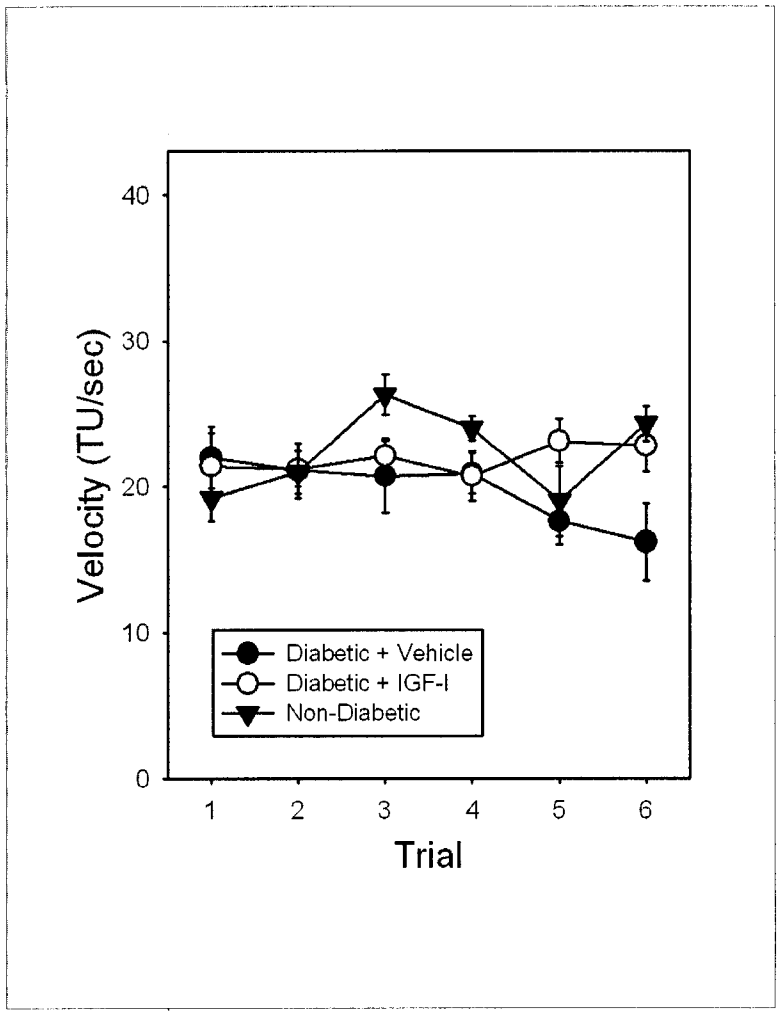

Fig. 2-7. Diabetes did not alter the velocity of rats swimming to a visible platform in a water maze test. There were no significant differences in mean velocity between any of the groups, nor did the velocity change over the trial for any of the groups. Symbols show means ± SEM. The data were analyzed by ANOVA with repeated measures. The symbols are means ± SEM.

motor or proprioceptive disturbances sufficient to impair performance in the water maze test. All rats reached the platform within 25.2 ± 4.0 (mean ± SEM) seconds. The latency for reaching the visible platform was shorter than for reaching the hidden platform on any place test day for all three groups. This showed that locating the visible platform was not by chance and most likely dependent on relatively competent visual capacity.

D. Hyperglycemia, Body Weights, Brain Weights and Hippocampus Weights in Diabetic Rats Treated with IGF-I: Rats were analyzed one day after the cued test (Table 2-1). Serum glucose levels were significantly increased in (Diabetic + Vehicle) and (Diabetic + IGF-I) *vs.* (Non-Diabetic) rats ($P < 0.001$). The 20 μg/rat/day IGF-I treatment did not reduce hyperglycemia in diabetic rats. Total body weight is a sensitive index of metabolic disturbance in diabetes. There was a significant reduction in total body weight in (Diabetic + Vehicle) and (Diabetic + IGF-I) *vs.* (Non-Diabetic) rats ($P < 0.001$).

There was a profound reduction in brain ($P < 0.001$) and hippocampus ($P < 0.001$) weights in (Diabetic + Vehicle) vs. (Non-Diabetic) rats. These differences persisted in the (Diabetic + IGF-I) rats.

VI. DISCUSSION

These data support the hypothesis that IGF-I treatment can prevent brain disturbances that contribute to impaired spatial learning/memory in diabetes. They further show that systemically administered IGF-I can support cognitive functions across the B-CNS-B. This may be of pharmacologic importance irrespective of mechanism,

TABLE 2-1. Effect of IGF-I treatment and diabetes on serum glucose, total body weight, brain weight and right hippocampus weight in diabetic rats

Parameter	Non-Diabetic	Diabetic + Vehicle	Diabetic + IGF-I
Rats per group	12	9	7
Glucose (mg/dL)	125 ± 11	515 ± 73*	495 ± 99*
Body weight (g)	541 ± 22	253 ± 35*	275 ± 32*
Brain weight (g)	2.16 ± 0.07	1.82 ± 0.09*	1.88 ± 0.04*
Hippocampus weight (mg)	93.6 ± 9.0	77.0 ± 5.8*	82.0 ± 9.0**

Rats were treated as described in Figs. 2-2 and 2-5. One day after completion of the visible platform test, rats were weighed, tail blood was removed for glucose assay, and the brain excised. The right hippocampus was isolated and weighed, and "brain weight" refers to the weight of the remainder of the brain absent hippocampi. Values are means ± S.D.

*$P < 0.001$ Nondiabetic vs. (Diabetic + Vehicle) or (Diabetic + IGF-I)

**$P < 0.02$ Nondiabetic vs. (Diabetic + IGF-I)

particularly because glycemic control only partially prevents the progression of neurological complications.

A. Water Maze Performance: IGF-I administration normalized *overall* performance in the water maze, a widely used measure of learning/memory in rats. Dissection of water maze performance leads to the consideration of factors such as sensory competence, task comprehension, motivation, acquisition of information, storage of information, information retrieval, utilization of information, and altered behavior dependent on competence of sensory-motor pathways. This discussion will comment on the effects of IGF-I, where pertinent, to some of these factors, and to potential mechanisms. However, it is not the intent to ascribe the actions of IGF-I at this time to specific learning/memory mechanisms, and this remains a matter for further study. For the sake of discussion, certain inferences have been drawn below. However, inferences based on the behavior of a rat should be approached with considerable caution and are subject to alternative interpretations. Independently of whether these inferences are correct, IGF-I treatment clearly prevented the impaired behavior of diabetic rats in the water maze.

1. Hidden Platform Test: Ultrastructural lesions in the hippocampus, amygdala and cortex are found in diabetes (Piotrowski et al., 1999). Diabetic rats displayed impaired behavior in both the hidden and visible platform tests. They had prolonged latencies to escape to a hidden platform in the Morris Water Maze (Fig. 2-2). This finding was in agreement with the observation of Biessels et al. (1996). The new observation is that IGF-I administration prevented the prolonged latencies to escape to the

hidden platform in diabetic rats (Fig. 2-2). This result shows that a hippocampal-based functional disturbance in diabetes can be prevented by a physiologic replacement dose of IGF-I.

Diabetic rats may have poorer comprehension and learning of the water maze task. The latency to escape to a hidden platform was progressively reduced beginning on the very first test day in nondiabetic rats (Fig. 2-2); this was associated with a reduced distance swum over successive days (Fig. 2-3). By contrast, the vehicle-treated diabetic rats required 3 days before their latency began to decline; this delay might be the result of an initial poorer comprehension of the task. Poorer comprehension in diabetic rats has been suggested by Biessels et al. (1996). Once the task was comprehended, the diabetic rats swam increasingly faster each day while searching for the hidden platform (Fig. 2-4). By contrast, IGF-treatment prevented the delay, and one interpretation is that the treatment overcame poor task comprehension in diabetic rats (Fig 2-2). On successive test days, their latency to escape to the hidden platform was reduced at the same rate as that of nondiabetic rats, and this was accomplished by a reduction in distance swum with no change in velocity. It is unlikely that streptozotocin toxicity was responsible for these disturbances in diabetic rats, because the induced disturbances were all prevented by IGF-I administration.

2. Visible Platform Test: All rat groups did better in the visible than the hidden platform test (see below). However, the mean latencies ($P < 0.014$)(Fig. 2-5) as well as distance swum ($P < 0.015$) (Fig. 2-6) of vehicle-treated diabetic rats remained significantly greater than that of the nondiabetic rats in the visible platform test. Thus, the

cued test uncovered evidence suggestive of mild impairment of comprehension, motivation, executive function or vision in diabetic rats. Alternatively, the diabetic rats might rely more on their memory of the position of the platform learned from visual cues in the hidden platform test, and not adjust as quickly as nondiabetic rats to the visible platform moved to a different position for each trial of the visible platform test. This is the case for rats with striatal lesions (McDonald and White, 1994), and diabetic rats may have striatal lesions (Piotrowski et al., 1999). In the visible platform test, hippocampal damage in diabetic rats might result in reduced capacity to extinguish the memory of the position of the former platform in swimming to the subsequent platform, leading to impaired latency and distance swam.

The mean latency and distance swum of IGF-treated diabetic rats were not significantly different from that of nondiabetic rats nor diabetic rats. The differences in latency and distance swam between diabetic and nondiabetic groups is much smaller in the visible than hidden platform test, and larger N values would be needed to test for an IGF-I effect.

3. Probe Test: Memory is somewhat impaired ($P < 0.05$) in diabetic rats (Biessels et al., 1996). Our own probe test was inconclusive. A significant difference was not found between (Nondiabetic) and (Diabetic + Veh) groups, but the $P = 0.18$ suggested that with a larger number of animals significance might be attained. It may be considered that these two groups were not trained to the same degree of competence in locating the hidden platform. On the last day of the hidden platform test, the (Nondiabetic) group located the platform with a mean latency of 35 sec, whereas the

(Diabetic + Veh) group had a significantly longer mean latency of 60 sec. Consequently, the (Diabetic + Veh) group might be expected to do poorer (spend less time in the defined area) in the probe test, but a significant difference was not observed. One possibility is that learning in the water maze is impaired to a greater extent than memory in diabetic rats, but further study is needed.

4. Sensorimotor Function: Although sensorimotor disturbances may be present in diabetic rats, such disturbances do not appear to be sufficiently advanced that performance in the water maze is impaired. For example, all rats, whether diabetic or nondiabetic, swam with the same velocity in the visible platform test (Fig. 2-7), demonstrating that proprioceptive and motor functions were not substantially impaired. The visible platform test does not require hippocampus-dependent spatial memory (McNamara and Skelton, 1993), but is dependent at least in part on the striatum (McDonald and White, 1994) and the visual pathway. All rats did better in the visible *vs.* the hidden platform test. For example, nondiabetic rats escaped to the hidden platform on the fifth test day in approximately 35 sec (Fig. 2-2) and swam a distance of 700 TU (Fig. 2-3), whereas their latency was reduced to under 20 sec (Fig. 2-5) and distance to 370 TU (Fig. 2-6) when the platform was visible. By contrast, the vehicle-treated diabetic rat group escaped to the hidden platform on the fifth day in about 60 sec (Fig. 2-2) and swam a distance of about 1200 TU (Fig. 2-3), whereas their latency was reduced to about 38 sec (Fig. 2-5) and distance to about 720 TU (Fig. 2-6) in the cued test. Because platform visibility improved latency and distance performance, the striatal and visual pathways apparently were at least partially intact in the diabetic rats.

Abnormalities in the electroretinogram are observed as early as 2 weeks, and, albeit not quantified, early signs of cataract formation are reported after 8 weeks of streptozotocin diabetes (Li et al., 2002a). Loss of retinal ganglion cells are reported as early as 4 weeks (Zeng et al., 2000). If mild visual impairment were present in diabetic rats, this may have been prevented by the IGF-I treatment. Treatment with des(1-3)IGF-I, an IGF-I analog, can prevent predegenerative biochemical changes in the retina of diabetic rats (Kummer et al., 2003).

B. IGF-I Administration Preserved Cognitive Function in Diabetes Independently of Hyperglycemia As Well As Smaller Brain and Total Body Weights: Neurological complications may arise in diabetes as a consequence of hyperglycemia. This could lead via aldose reductase to elevated polyol levels in the peripheral nervous system, but, disappointingly, peripheral neuropathy has not been prevented in the many clinical trials with aldose reductase inhibitors. One may not exclude the potential contribution of nonenzymatic glycation that might alter the function of proteins. Systemic hyperglycemia is evident within one day of administration of streptozotocin under the conditions of the present study (Wuarin et al., 1996; Zhuang et al., 1996). A persistent hyperglycemia is observed in measurements at 1, 4, 7, 14, and 28 days. Glucose levels remained elevated in the serum of diabetic rats at 12 weeks in the present study, and hyperglycemia was not attenuated by IGF-I treatment (Table 2-1), although loss of performance in the water maze was prevented. A loss of IGF activity, rather than hyperglycemia, may confer the primary risk for cognitive disorder in diabetes, albeit long-term hyperglycemia, microangiopathy and cerebrovascular hemorrhage may

further aggravate this risk in chronic disease.

The hippocampus and the remainder of the brain were approximately 15 % smaller in weight in diabetic relative to nondiabetic rats (Table 2-1). The content of total transcripts is reduced in brain and hippocampus within two weeks streptozotocin diabetes, and smaller brain weight is most likely due to loss of protein (Wuarin et al., 1996). Apoptosis may be present (Li et al., 2002b). It was somewhat astonishing that IGF-I prevented the impairment of performance in the water maze of the diabetic rats without ameliorating these substantial weight differences. Diabetic rats had significantly lower body weights than nondiabetic rats, and IGF-I treatment did not prevent weight loss (Table 2-1); weight loss is a sensitive index of the metabolic disturbance in diabetes. These data show that impaired cognitive performance was unlikely to be the consequence of loss of brain or body weight *per se*. Furthermore, this indicates that IGF-I was unlikely to have preserved cognitive function primarily as a consequence of having improved the overall anabolic state in the brain. Rather, these data suggest that IGF-I selectively preserved the pathways that contribute to performance in the water maze. Hippocampal-based memory functions are similar between rats and humans (Bunsey and Eichenbaum, 1996). The difference in brain weights between diabetic and nondiabetic rats might be due to hyperglycemia or other causes.

The capacity of IGFs to prevent neurological disturbances despite hyperglycemia in diabetic rats is observed in the peripheral as well as central nervous system. Impaired nerve regeneration and hyperalgesia are prevented or reversed by administration of IGF doses as small as 5 μg/rat/day in type 1 diabetic rats independently of hyperglycemia and

weight loss (Ishii and Lupien, 1995; Zhuang et al., 1996). Neuroaxonal dystrophy is a term used to described the characteristic ultrastructural lesions encountered in clinical nerve specimens, and IGF-I administration can reverse neuroaxonal dystrophy in autonomic nerves of rats diabetic for six months (Schmidt et al., 1999).

The small 20 μg IGF-I per day dose did not prevent hyperglycemia in the diabetic rats. However, pharmacological doses much higher than the replacement IGF-I dose used in this study can reduce hyperglycemia. The daily production of IGF-I in adult rat liver is approximately 31 μg/day (Schwander et al., 1983; Scott et al., 1985), and the dose used in this study was 20 μg/rat/day. IGF-I and insulin have similar 3-dimentional conformation. At doses several-fold higher than the daily production, IGF-I can cross-occupy insulin receptors and reduce hyperglycemia.

C. Systemically Administered IGF-I Preserves Cognitive and Other Central Nervous System Functions: The uptake of circulating IGF-I into CSF is saturable, suggesting that there is an uptake carrier at the B-CNS-B (Armstrong et al., 2000). This uptake shows independence from the IGF type 1 receptor, type 2 receptor, as well as IGF sequestration to IGF binding proteins (Pulford and Ishii, 2001). It is calculated that the normal circulating concentration of IGF-I may contribute substantially to the concentration of IGF-I detected in CSF, and this provides critical support for the hypothesis that circulating IGF supports ongoing functions in the adult mammalian central nervous system.

The subcutaneous administration of IGF-I can prevent poor water maze performance in diabetes, showing that IGF-I can preserve brain function across the B-

CNS-B. The impermeability of the B-CNS-B is well maintained in diabetes, albeit carrier-mediated transport of some metabolites may be altered (for review see Mooradian, 1997). Because IGF is taken up from blood into CSF, the clinical risk associated with the need to drill an access hole through the skull to deliver a neurotrophic IGF protein (7.5 kDa) may be averted.

VII. ACKNOWLEDGMENTS:

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CHAPTER 3

Biochemical Pathology and Effects of Insulin-Like Growth Factor-I Administration in a Rat Model of Diabetic Dementia

Biochemical Pathology and Effects of Insulin-Like Growth Factor-I Administration in a Rat Model of Diabetic Dementia

Sean B. Lupien and Douglas N. Ishii

I. ABSTRACT: Atrophy of diabetic brain is shown by MRI and CT scans. The biochemical pathology in diabetic brain atrophy is not understood. Since insulin-like growth factor (IGF) levels are reduced in diabetes and can cross the blood-CNS-barrier, we tested the hypothesis that subcutaneous IGF-I administration can prevent CNS disturbances in diabetes independently of hyperglycemia. Adult rats were rendered diabetic with streptozotocin for 12 weeks, and *s.c.* Alzet pumps continuously released vehicle (D+Veh) or 20 μg/rat/day IGF-I (D+IGF) during the final 8 weeks. Brains were excised, weighed, and the biochemical pathology was investigated. Wet brain weights ($P < 0.001$), 18S rRNA per brain ($P < 0.002$) and poly(A)$^+$ RNA ($P < 0.04$) per brain were significantly reduced in (D+Veh) vs. Nondiabetic rats, and IGF-I treatment had no effect. The (mg protein)/(wet weight brain) as well as (mg protein)/(brain) were significantly reduced in (D+Veh) vs. Nondiabetic rats ($P < 0.001$), and IGF-I treatment prevented these reductions (D+IGF-I) vs. (D+Veh) ($P < 0.03$) independently of ongoing hyperglycemia. These data show that s.c. IGF-I administration can prevent loss of protein content in brain as well as cognitive dysfunction in diabetes despite ongoing hyperglycemia. Brain atrophy may be due in part to reduced protein, mRNA and rRNA contents.

II. INTRODUCTION:

Brain atrophy in diabetes is observed by magnetic resonance imaging (Dejgaard et al., 1991; Araki et al., 1994) and computerized tomography (Soininen et al., 1992). Autopsy of diabetic patients reveals structural lesions, including axonal loss and the degeneration of ganglion and cortical neurons (Reske-Nielsen et al., 1965). The brain ventricles are enlarged, showing shrinkage of brain matter (Dejgaard et al., 1991; Araki et al., 1994). Scattered loss of anterior horn neurons has also been described (DeJong, 1977; Jakobsen and Sidenius, 1980) The early onset and high incidence of central electrophysiological disturbances suggest that encephalopathy is an accompanying complication of diabetes (Pietravalle et al., 1993; Di Luca et al., 1999).

The streptozotocin (STZ) diabetic rat is a widely used model of type 1 diabetes with hypoinsulinemia, hyperglycemia and reduced body weight. Structural lesions occur in the brain of STZ rats (Jakobsen et al., 1987; Lincoln et al., 1989; Tay and Wong, 1992) and central conduction velocity is reduced (Carsten et al., 1989; Terada et al., 1993).

It has been proposed that a decline in insulin-like growth factor (IGF) activity contributes to diabetic neurological disturbances (Ishii, 1995). Circulating IGF-I levels are reduced in Type 1 and Type 2 diabetic patients (Tan and Baxter, 1986) and such reduction is greater in patients with neuropathy than without (Migdalis et al., 1995; Guo et al., 1999). IGF-I and IGF-II gene expression are reduced in the peripheral nerves and spinal cord, and IGF-II gene expression is reduced in the brain of STZ rats (Ishii et al., 1994; Wuarin et al., 1994; Wuarin et al., 1996; Zhuang et al., 1997). IGF-II is the predominant IGF in the adult rat brain. The administration of low replacement doses of

IGF-I or IGF-II prevents peripheral neuropathy in type 1 and type 2 diabetic rats independently of hyperglycemia (Ishii and Lupien, 1995; Zhuang et al., 1996; Zhuang et al., 1997; Schmidt et al., 1999). IGF-I can cross the B-CNS-B and a substantial fraction of IGF-I in the CNS is thought to arise from the circulation (Reinhardt and Bondy, 1994; Armstrong et al., 2000). Because circulating IGF-I levels are reduced in diabetic patients, diminished IGF activity may contribute to central as well as peripheral neurological disturbances in diabetes.

The biochemical pathology in the brain of diabetes is unknown. It is of interest to characterize the biochemical pathology in diabetes so that brain atrophy may be better understood. It is also of interest to determine whether systemically administered IGF-I can prevent biochemical pathology in the diabetic brain in spite of unabated hyperglycemia.

III. HYPOTHESES:

The purpose of this study was to test the following hypotheses: i) a decline in total protein content is associated with brain atrophy in diabetes, ii) a decline in total mRNA content is associated with brain atrophy in diabetes, iii) a decline in ribosomal RNA content is associated with brain atrophy in diabetes, iv) IGF-I can prevent loss of brain protein content associated with diabetic brain atrophy, v) systemically administered IGF-I is effective across the B-CNS-B, and vi) IGF can prevent loss of brain protein content independently of unabated hyperglycemia.

IV. MATERIALS AND METHODS:

A. Animals: Rats used in this study are the same rats used for the cognition study in Chapter 2. Briefly rats were randomly assigned to one of three treatment groups. Two groups were rendered diabetic for 12 weeks and received either 20 μg/day/rat of IGF-I (diabetic + IGF-I) or vehicle (diabetic + vehicle) for the final 8 weeks of diabetes. Another group of rats was untreated (non-diabetic). After the last day of the Morris Water Maze visible platform test (12 weeks of diabetes), rats were weighed, tail blood withdrawn for glucose assay and rats were euthanized. The brains were dissected, the hippocampus was removed and the brain and right hippocampus were weighed. Tissues were frozen in liquid nitrogen and stored at -70°C.

B. Protein Quantification: The left brain was kept frozen on dry ice, added to 10 mls of homogenization buffer (10 mM Tris-acetate and 5 mM EDTA, pH7.4, containing the protease inhibitors leupeptin (10 μg/ml), chymostatin (10 μg/ml), pepstatin A (10 μg/ml), and 0.1 mM phenylmethylsulfonyl fluoride) and homogenized using a Tissue Tearer (Fisher, 15-338-55). Samples were frozen on dry ice in aliquots and stored at -70°C. An aliquot was thawed on ice water to reduce protein degradation and the protein concentration was quantitated using the colorimetric DC Protein Assay (Bio-Rad, 500-0112). Bovine serum albumin was used for the standard.

C. RNA Purification: RNA from the right brain was isolated by the guanidinium thiocyanate procedure of Chomczynski and Sacchi (1987). RNA concentration and purity were estimated by absorbance at 260 and 280 nm. RNA yield was linear with tissue weight, permitting calculation of total RNA content per tissue.

D. RNA Slot Blots: Equivalent amounts of total RNA were incubated for 15 min at 68°C in 14 % formaldehyde and 7.5X SSC (1X SSC is 150 mM NaCl and 15 mM sodium citrate, pH7), rapidly cooled on ice, and directly loaded (6 μg in duplicate) onto supported nitrocellulose using a slot blot filtration device (Bio-Rad, Bio-Dot SF). To normalize between blots, four samples were loaded in triplicate onto each blot. Nitrocellulose blots were baked at 80°C for 45 min under vacuum and cross-linked to RNA using UV light at 0.12 J/cm^2 (Stragene, La Jolla, CA). Various concentrations (0, 1, 2, 4, 6, 8, 10 μg) of an RNA standard were included on each slot blot to ensure that exposure to a phosphoscreen was in the linear range.

E. Preparation of Probes: Oligo(dT)$_{18}$ (synthesized by Marcro Molecular Resources, Fort Collins, CO) was end-labeled with radioactivity using T4 Kinase (Invitrogen) and gamma-^{32}P-ATP (Amersham). Single-stranded antisense 18S rRNA (Ambion, 7339) was labeled with radioactivity by the Taq Primer extension method. 18S rRNA plasmid, alpha-^{32}P-dCTP (Amersham), T7 primer, and dNTPs were used in primer extension with Taq DNA polymerase (Promega) and subjected to 45 cycles of thermocycling (95°C for 1 min, 40°C for 1 min, and 72°C for 1 min) in a Perkin Elmer 480 Thermo Cycler followed by a 7 min final extension at 72°C and a hold at 4°C. Both probes were purified to remove unincorporated nucleotides in a Bio-Spin P-30 Chromatography column (Bio-Rad).

F. Hybridization to RNA: Nitrocellulose blots were hybridized simultaneously in the same tube to the Oligo(dT)$_{18}$ probe for 1 hour and then washed in 2X SSC. Blots were subsequently exposed to a phosphorscreen, and scanned on a PhophorImager

scanner (Molecular Dynamics, Sunnyvale, CA). The blot was stripped of probe following the method in Sambrook et al., 2000 (50% formamide, 0.1X SSC, 0.1% SDS at 68°C overnight) and verified by phosphoimager exposure. The nitrocellulose blots were hybridized to the 18S rRNA probe for 1 hour and then washed using a procedure to detect low abundance transcripts (Sambrook et al., 2000). Blots were subsequently exposed to a phosphorscreen, and scanned on a PhophorImager scanner (Molecular Dynamics, Sunnyvale, CA).

G. Quantification of RNA Slot Blots: Images of the scanned slot blots were quantified using Molecular Dynamics' ImageQuant V5.1 software. The VUC, or densitometric volume under the curve, was quantified in relative arbitrary units for each band on the slot blots. The VUC for slots loaded only with buffer were averaged and deducted as background from each sample on the slot blot. Poly(A)+ RNA per total brain (mg) was calculated for each rat sample by: oligo(dT) VUC (averaged for duplicate samples) x (total volume of purified RNA/volume prepared for slot blot) x (volume of tissue homogenization buffer/volume used for RNA purification) x (total volume of slot blot prepared RNA/volume loaded on slot blot) x ((left brain weight + right brain weight)/right brain weight). Poly(A)+ RNA per wet brain weight was calculated by dividing the poly(A)+RNA per brain by wet brain weight (mg/gm). Ribosomal RNA per total brain (mg) and rRNA per wet brain weight (mg/gm) were calculated using the same method as for poly(A)+RNA per total brain and per wet brain weight except the VUC was for 18S rRNA. Poly(A)+RNA per ribosomal RNA was calculated by dividing the oligo(dT) VUC by the 18S rRNA VUC. The ratio for duplicate samples was averaged.

The group means were then computed.

H. Statistical Analysis: Statistical analysis was made with computer software using Newman-Keuls post hoc test of means (CSS Statistica; StatSoft, Tulsa, OK).

V. RESULTS:

A. Protein per Brain and Protein per Wet Brain Weight Are Reduced in Diabetes: Effects of IGF-I: The content of total brain protein was significantly reduced in the (diab+veh) or (diab + IGF-I) groups versus the (non-diabetic) group ($P < 0.001$) (Fig 3-1). However, treatment with IGF-I significantly increased the total brain protein content in the (diab + IGF-I) group versus (diab + veh) group ($P = 0.032$). The content of brain protein per wet brain weight was also reduced in the (diab + veh) group versus the (non-diabetic) group ($P < 0.002$) (Fig 3-2). IGF-I treatment prevented a loss of brain protein content per wet weight of brain in the (diab + IGF-I) group versus the (diab + veh) group ($P < 0.03$) and there was no difference between the (non-diabetic) group and the (diab + IGF-I) group.

B. Poly(A)+ RNA per Brain Is Reduced in Diabetes: The content of total poly(A)+ RNA per brain was significantly reduced in the (diab + veh) group versus the (non-diabetic) group ($P = 0.036$) (Fig 3-3). IGF-I treatment may have ameliorated the significant reduction in poly(A)+ RNA per brain in diabetes since there is no difference between the (diab + IGF-I) group and the (non-diabetic) group. However, IGF-I did not significantly prevent a reduction in poly(A)+ RNA per brain in the (diab + IGF-I) group

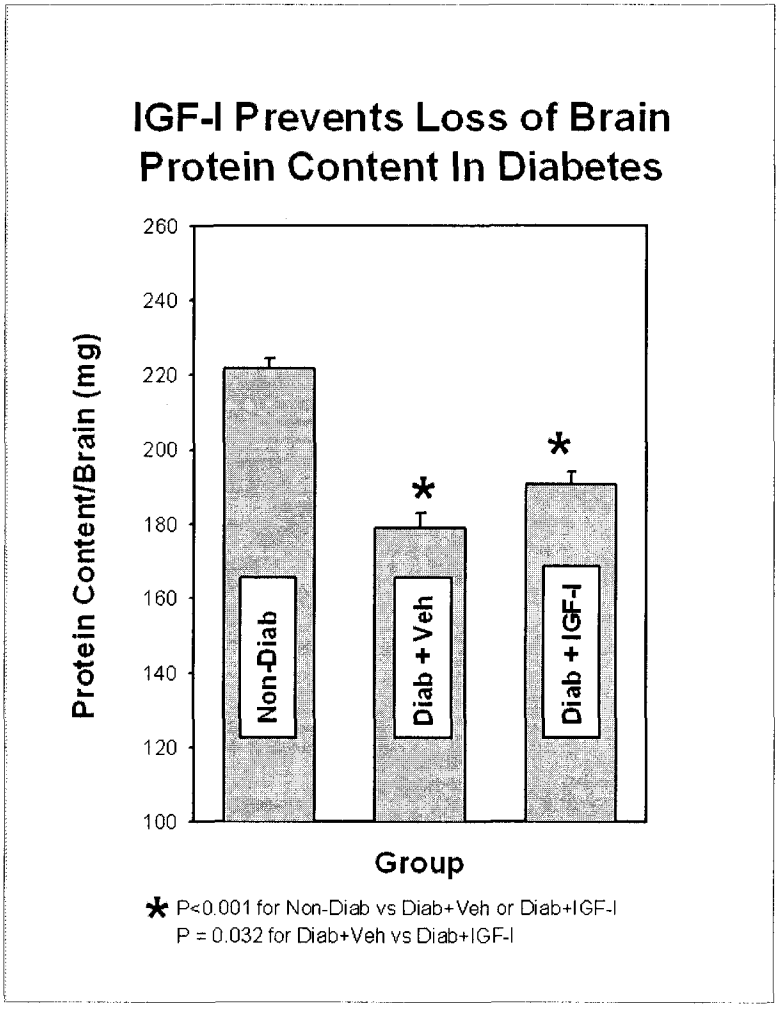

Fig. 3-1. IGF-I administration partially prevents a loss of brain protein content in diabetes. Rats were rendered diabetic by STZ for 12 weeks and then implanted subcutaneously with osmotic minipumps that released either 20 μg/day IGF-I (Diabetic + IGF-I) or vehicle (Diabetic + Vehicle) for the final 8 weeks. After 12 weeks of diabetes, the left brain was homogenized in buffer containing protease inhibitors, and protein content was determined with a colorimetric enzymatic assay. Total brain protein content was reduced in the (Diab+Veh) or the (Diab+IGF-I) vs. the (Non-Diab) group ($P<0.001$). IGF-I administration partially prevented this reduction in the (Diab+IGF-I) vs. the (Diab+Veh) group ($P<0.032$). Values are means $\pm$ S.E.M. Newman-Keuls post-hoc test of the means was used for statistical analysis.

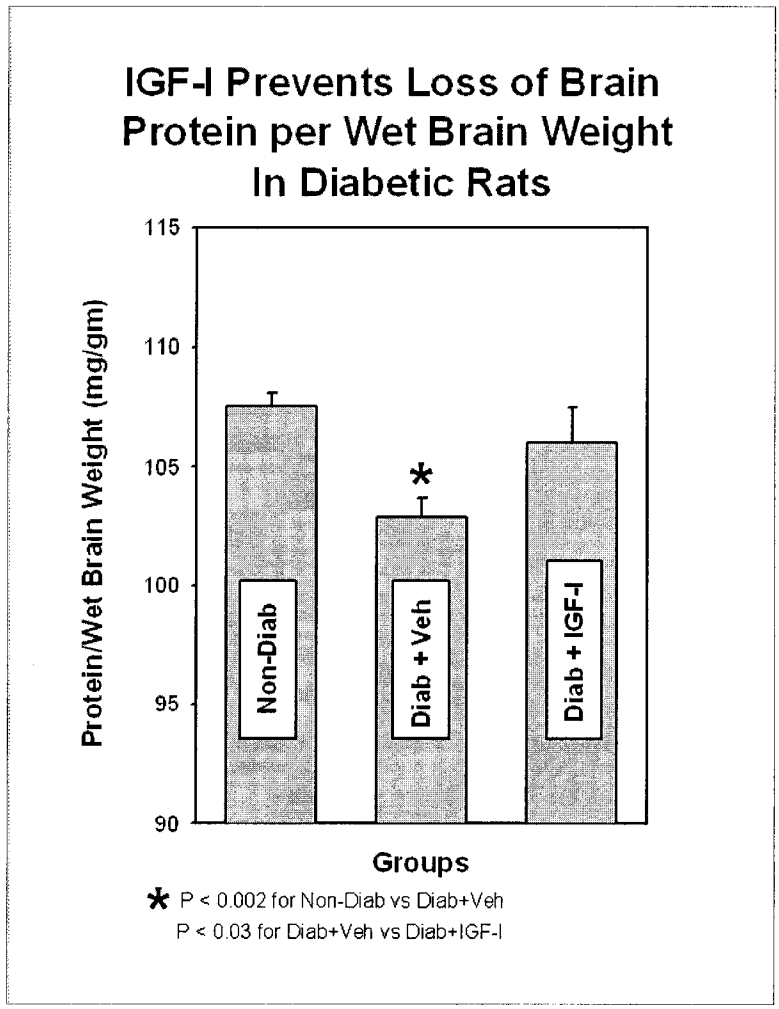

Fig. 3-2. IGF-I treatment prevented loss of brain protein content per wet weight of brain in diabetic rats. Protein per wet brain weight was reduced in the (Diab+Veh) vs. the (Non-Diab) group ($P<0.002$). IGF-I treatment almost completely prevented this reduction in the (Diab+IGF-I) vs. the (Diab+Veh) group ($P<0.03$). Values are means $\pm$ S.E.M. Newman-Keuls post-hoc test of the means was used for statistical analysis.

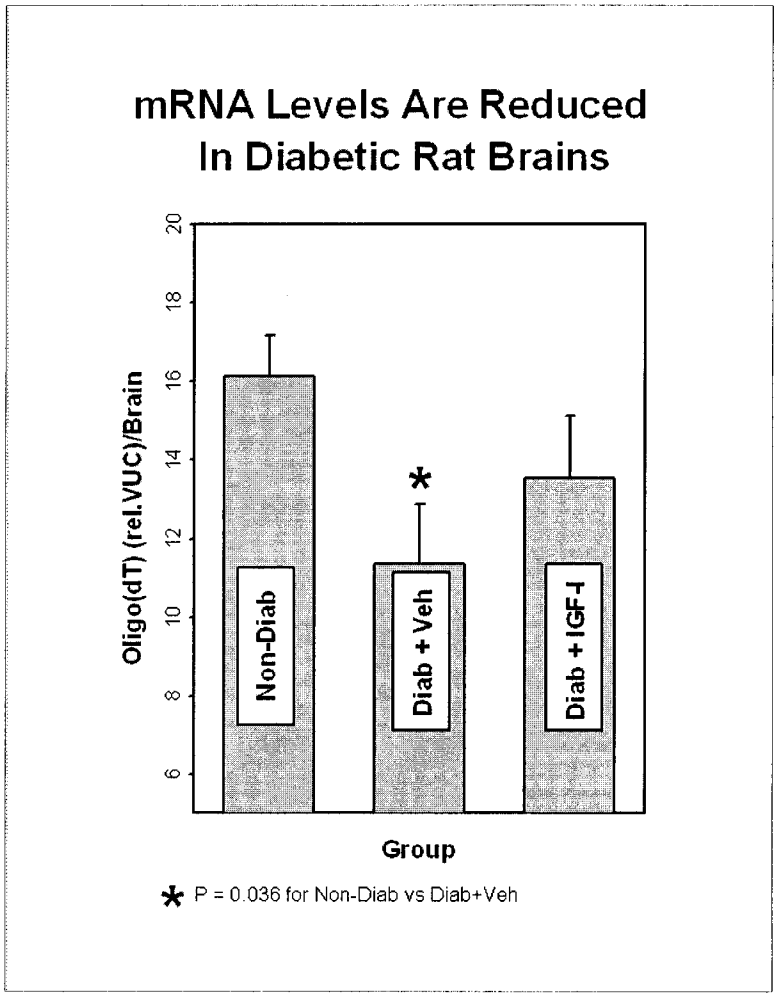

Fig. 3-3. Poly(A)+ RNA content was reduced in diabetic rat brains. Total RNA was isolated from the right brain using the guanidinium thiocyanate procedure (Chomczynski and Sacchi, 1987). Equivalent amounts of RNA (in duplicate) were loaded onto nitrocellulose slot blots, and hybridized to ^{32}P-labeled oligo(dT)$_{18}$ to detect total poly(A)+ RNA. A Molecular Dynamics phosphoimager connected to a computer was used to capture hybridization images, and relative pixel densities were quantified. Poly(A)+ RNA content per brain was reduced in the (Diab+Veh) vs. the (Non-Diab) group ($P<0.036$). IGF-I administration may have partially ameliorated this reduction because there was no significant difference between the (Diab+IGF-I) vs. the (Non-Diab) group, albeit the (Diab+IGF-I) group was not significantly different from the (Diab+Veh) group. Values are means $\pm$ S.E.M. Newman-Keuls post-hoc test of the means was used for statistical analysis.

versus (diab + veh) group. A power calculation showed that about 75 animals per group would be required to achieve 90% certainty that there would be a significant difference at $P < 0.05$. There was no difference in poly(A)+RNA per wet weight of brain in any of the groups after IGF-I treatment.

C. Ribosomal RNA per Brain And per Wet Brain Weight Are Reduced in Diabetes: The content of rRNA per brain was significantly reduced in the (diab + veh) or the (diab + IGF-I) groups versus the (non-diabetic) group ($P < 0.014$) (Fig 3-4). IGF-I treatment had no effect on diabetes since there was no difference between the (diab + IGF-I) group and the (diab + veh) group. The content of rRNA per wet brain weight showed a strong trend towards reduction in the (diab + veh) group versus the (non-diabetic) group ($P = 0.057$) (Fig 3-5). A power calculation showed that about six more animals per group would be required to achieve 90% certainty that there would be a significant difference at $P < 0.05$. IGF-I treatment might have ameliorated the reduction in rRNA content per wet brain weight in diabetes since there was no difference between the (diab + IGF-I) group and the (non-diabetic) group. However, IGF-I did not significantly increase rRNA content per wet brain weight in the (diabetic + IGF) group versus the (diab + veh) group. A power calculation showed that about 100 animals per group would be required to achieve 90% certainty that there would be a significant difference at $P < 0.05$.

D. Poly(A)+ Content per Total RNA Is Not Altered in Diabetes: There was no difference in poly(A)+RNA per rRNA between any of the groups. This was probably due to the decrease in both poly(A)+ RNA and 18S rRNA content in diabetes.

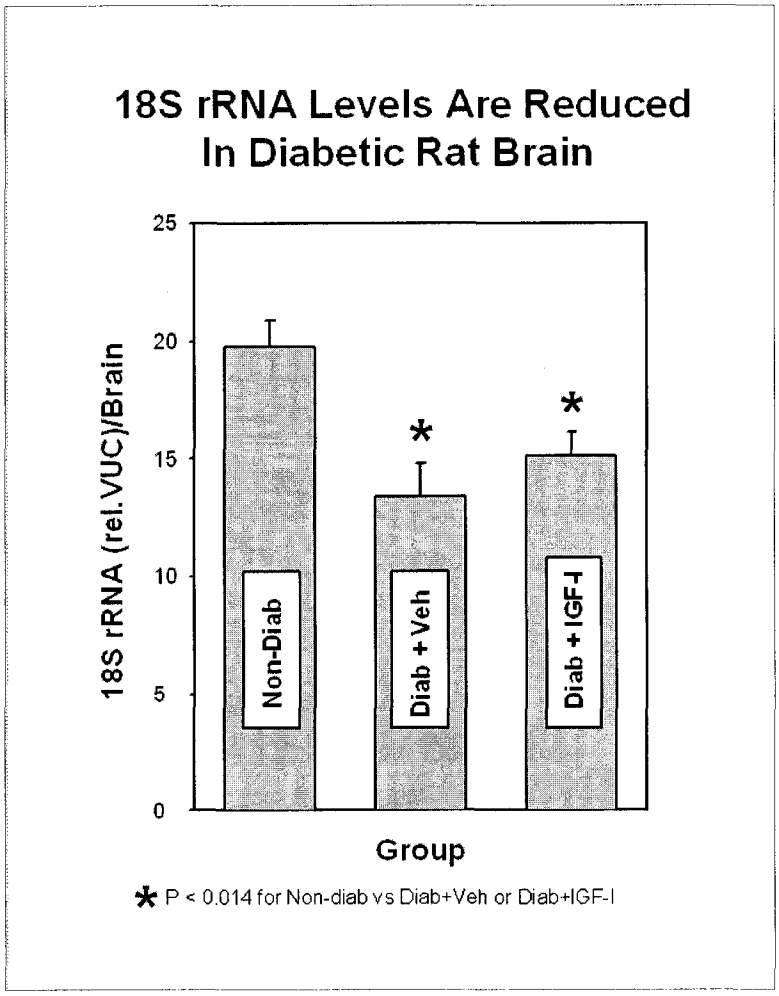

Fig 3-4. 18S rRNA content was reduced in diabetic rat brain. Slot blots were washed and subsequently hybridized to a ^{32}P-labeled single-stranded anti-sense 18S rRNA cDNA (probe previously characterized by Northern blots). A Molecular Dynamics phosphoimager connected to a computer was used to capture hybridization images, and relative pixel densities were quantified. 18S rRNA levels per brain were reduced in the (Diab+Veh) or the (Diab+IGF-I) vs. the (Non-Diab) group ($P<0.014$). IGF-I administration had no effect on diabetes. Values are means $\pm$ S.E.M. Newman-Keuls post-hoc test of the means was used for statistical analysis.

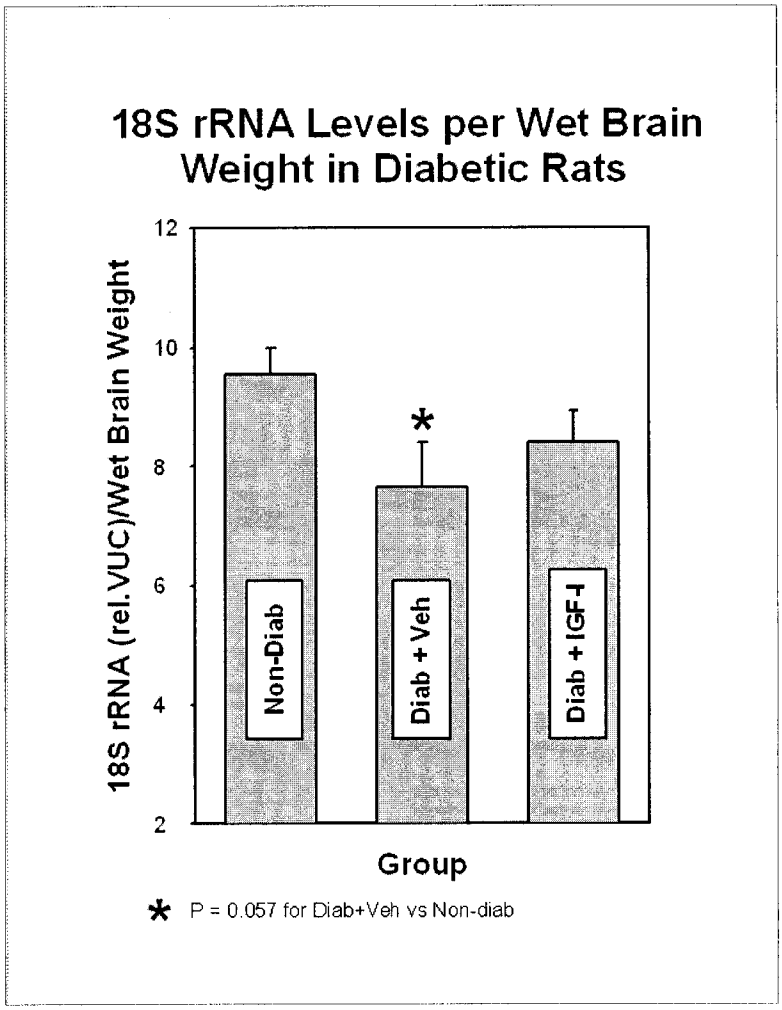

Fig 3-5. 18S rRNA levels per wet brain weight showed a strong trend in reduction in the (Diab+Veh) vs. the (Non-Diab) group (P<.057). There were no significant differences between the (Diab+IGF-I) vs. either the (Non-diab) or (Diab+Veh) groups. Values are means $\pm$ S.E.M. Newman-Keuls post-hoc test of the means was used for statistical analysis.

VI. DISCUSSION:

In chapter 2, it was shown that rats diabetic for 12 weeks had cognitive impairments that were prevented by subcutaneous IGF-I treatment. In spite of IGF-I treatment, brain atrophy was not prevented in diabetic rats as shown by reduced wet brain weights. It is possible that reduced brain weight could be due to loss of water. However, these data show that loss of brain weight in diabetes is at least due in part to reduced amounts of protein, messenger RNA, and ribosomal RNA. Systemically administered IGF-I can cross the B-CNS-B and partially prevent reduced brain protein content in diabetes.

A. Brain Atrophy in Diabetes Is Due in Part to Reduced Protein, Poly(A)+ RNA, and Ribosomal RNA Content and IGF-I Partially Prevented Reduced Protein Content: These data extend present understanding of the biochemical pathology in the brain associated with brain atrophy in diabetes. To the best of our knowledge, this is the first demonstration of reduced protein, mRNA, and rRNA contents in diabetic brain atrophy.

1. Protein: In rats diabetic for 12 weeks, there is a significant reduction in protein content per brain in the (diab + veh) group versus the (non-diabetic) group (Fig. 3-1) showing that brain atrophy is not entirely due to loss of water. A loss of total protein content is associated with diabetic brain atrophy. The results also show reduced protein content per wet weight of brain in the (diab + veh) group versus the (non-diabetic) group (Fig. 3-2) meaning that protein synthesis and/or protein stability is reduced in diabetes. Protein synthesis is necessary for learning and memory (Quevedo et al., 1999; Vianna et

al., 2001; Naghdi et al., 2003) which may explain the cognitive impairments observed in diabetic rats discussed in Chapter 2.

IGF-I treatment partially prevented a reduction in protein content per brain in diabetic rats. Protein content in the (Diab + IGF-I) group was significantly higher than the (diab + veh) group (Fig. 3-1). However, IGF-I treatment did not completely prevent reduced brain protein content since the (diab + IGF-I) group was significantly reduced versus the (non-diabetic) group. IGF-I treatment completely prevented a reduction in protein content per wet brain weight. Protein content per wet brain weight in the (diab + IGF-I) group was significantly higher than the (diab + veh) group (Fig. 3-2) and there was no difference between the (diab + IGF-I) group versus the (non-diabetic) group. Increased protein content per wet brain weight could be due in part to increased protein synthesis. IGF-I supports protein synthesis in cultured human neuroblastoma cells associated with neurite outgrowth (Recio-Pinto and Ishii, 1984).

2. Poly(A)+ RNA: Protein synthesis requires in part messenger RNA that provides the code for translation of primary peptides. A reduction in poly (A)+ RNA could result in a decrease in protein content since there would be fewer transcripts. It has been shown that many mRNA sequences in the brain have small or no 3-prime poly-adenylation sequences (Chaudhari and Hahn, 1983). The probe used in these experiments was oligo(dT)18 and will not hybridize to mRNA without a polyadenylation sequence. However, the probe will recognize poly(A)+ RNA which is a sub-population of total mRNA. Therefore, poly(A)+ RNA is referred to here rather than mRNA. We found that total poly(A)+ RNA per brain in (diab + veh) rats was significantly reduced versus (non-

diabetic) rats (Fig. 3-3). This could in part explain reduced protein content per brain in diabetic rats.

It has previously been shown in STZ rats that the level of poly(A)+ RNA per wet brain weight is reduced after two weeks of diabetes (Wuarin et al., 1996). Our data show no difference in mRNA per wet brain weight after 12 weeks of diabetes in the (diab + veh) group versus the (non-diabetic) group (Fig. 3-4). There are several possibilities to explain this. Perhaps a certain subset of IGF-dependent brain cells are most afflicted by diabetes. Over two weeks of diabetes, they might produce fewer transcripts resulting in reduced poly(A)+ RNA per wet brain weight. Ten weeks later, the same subset of cells may have degenerated completely. A loss of these cells would result in a higher level of Poly(A)+ RNA per remaining wet brain weight after 12 weeks of diabetes than after 2 weeks. Another possible reason we do not detect a significant reduction in Poly(A)+ RNA per wet brain weight is that compensatory mechanisms come into play in longer duration diabetes.

IGF-I treatment had no significant effect on mRNA levels in the brains of diabetic rats. Multiple factors contribute to protein synthesis. Smaller, undetectable effects of IGF-I treatment on some factors that contribute to protein synthesis such as total mRNA content or rRNA content could lead to a detectable overall increase in protein synthesis. In fact, IGFs can elevate tubulin and neurofilament mRNAs during neurite outgrowth in SH-SY5Y neuroblastoma cells (Mill et al., 1985; Fernyhough et al., 1989) that can lead to increased synthesis of major cytoskeletal proteins. It is possible that when studying the whole brain, large differences in small populations of mRNA could be masked.

3. 18S rRNA: 18S rRNA is a major component of the 40S ribosomal subunit. The 40S subunit must bind mRNA before the 60S ribosomal subunit, which is partly comprised of 28S rRNA, can attach and begin translation to generate a primary peptide. Therefore, a reduction in 18S rRNA could impair protein synthesis by decreasing the amount of protein synthesis machinery. We found that 18S rRNA content per brain was significantly reduced in the (diab + veh) group versus the (non-diabetic) group (Fig. 3-5). Although the content of 18S rRNA per wet brain weight was not significantly reduced, there was a strong trend towards a reduction in the (diab + veh) group versus the (non-diabetic) group (Fig. 3-6). IGF-I treatment had no effect on 18S rRNA content in diabetic rats. For the same reasons discussed about mRNA, small but insignificant preventions of reduced 18S rRNA by IGF-I treatment may add up to a significant prevention in reduced protein content.

The combined reduction of 18S rRNA as well as poly(A)+ RNA may explain the reduced protein content per wet brain weight in diabetes. Perhaps with a larger number of animals, the reduction in 18S rRNA per wet brain weight would be significant. IGF-I can increase RNA synthesis in cultured human neuroblastoma cells (Recio-Pinto and Ishii, 1984), and loss of brain IGF in diabetes may potentially explain directly the loss of brain RNA content.

Although IGF-I did not significantly increase wet brain weight in diabetic rats, cognitive dysfunction was prevented as shown in Chapter 2. This suggests that IGF-I selectively preserved the learning and memory machinery in diabetic rats. Reinforcement for this interpretation is provided by the experiments in Chapter 5. It may be difficult to

detect many biochemical alterations in the learning and memory machinery when whole brain homogenate is examined. This study looked at biochemical pathology in the whole brain and alterations in machinery for other functions would be included. Larger numbers of animals or different methods may allow detection of significant differences of the biochemistry examined in this study.

B: Hyperglycemia: IGF-I administration partially prevented reduced protein content per brain and completely prevented reduced protein per wet brain weight in diabetic rats despite unabated hyperglycemia. IGF-I treatment had no effect on severity of hyperglycemia or reduced body weight in diabetic rats (Table 2-1). Body weight is a sensitive index of poor metabolic control. IGF-I or IGF-II administration, despite unabated hyperglycemia in experimental diabetes, can prevent or reverse various neurological disturbances including impaired sciatic nerve regeneration (Ishii and Lupien, 1995; Zhuang et al., 1996), hyperalgesia (Zhuang et al., 1996; Zhuang et al., 1997), neuraxonal dystrophy (Schmidt et al., 1999), abnormal gene expression in brain (Armstrong et al., 2000), and impaired learning/memory (Lupien et al., 2003). These observations together are of clinical interest, because they support the hypothesis that IGF administration can prevent peripheral diabetic neuropathy as well as central neurological disturbances even in poorly controlled diabetic patients.

VII. ACKNOWLEDGMENTS:

This work was supported in part by Grant R49/CR811509 from the Center for Disease Control and Prevention (to D.N.I.) and Predoctoral Fellow support to S.B.L. by

National Institute of Health contract grant number 1T32NS43115.

CHAPTER 4

Loss of Neuroretinal Cells and Effects of Insulin-like growth factor-I Administration in Diabetic Rats

The experiments in this chapter will be submitted for publication (manuscript in preparation). The work was a collaborative effort by the authors listed on the following page. I was responsible for providing the diabetic rats and dissecting and fixing the tissue. Gail Seigel and her group performed all the immunohistochemistry.

Loss of Neuroretinal Cells and Effects of Insulin-like growth factor-I Administration in Diabetic Rats

Gail M. Seigel, Sean B. Lupien, Lorrie M. Campbell, Douglas N. Ishii

I. ABSTRACT: Atrophy of the brain occurs in diabetes that may lead to dementia. The biochemical pathology in diabetic brain includes reduced protein content as shown in Chapter 3. Brain atrophy may also be due to apoptosis of brain cells and it is not known whether IGF can prevent loss of neurons in diabetes. Since insulin-like growth factor (IGF) levels are reduced in diabetes and can cross the blood-CNS-barrier, we tested the hypothesis that subcutaneous IGF-I administration can inhibit death of neuroretinal cells in diabetic rats by examining expression of pro-apoptotic markers. In diabetic rat retina, the number of TUNEL-immunoreactive cells increased approximately 6-fold in the photoreceptor layer ($P < 0.001$) and 8-fold in the inner nuclear layer ($P < 0.001$); phospho-Akt (Thr 308) immunoreactivity increased 8-fold in the ganglion cell layer ($P < 0.001$) and 3-fold in the inner nuclear layer ($P < 0.01$). Subcutaneous IGF-I treatment significantly reduced the number of TUNEL ($P < 0.001$) and phospho-Akt immunoreactive retinal cells ($P < 0.05$) in diabetic rats approximately to the level of the non-diabetic group. Elevated TUNEL and phospho-Akt immunoreactivities were localized to distinct cell layers in the retina of diabetic rats. Early intervention with systemic IGF-I reduced the presence of pro-apoptotic markers indicative of neuroretinal

cell death, despite ongoing hyperglycemia and weight loss. The eye is a special sensory organ, and these data show that cell loss in the nervous system, even in uncontrolled diabetes, can be prevented by IGF-I administration.

II. INTRODUCTION

In chapter 2, it was shown that rats diabetic for 12 weeks had cognitive impairments that were prevented by subcutaneous IGF-I treatment. In spite of IGF-I treatment, diabetic rats also had brain atrophy shown by reduced wet brain weights (Table 2-1). In chapter 3, it was shown that diabetic rats had reduced brain protein content that is associated with brain atrophy and this was prevented by subcutaneous infusion of IGF-I.

Atrophy of the brain in diabetes might also be caused by loss of cells. Increased TUNEL positive cells are detected in the hippocampus of Type 1 diabetic rats (Li et al., 2002b) and IGF-I and IGF-II levels are reduced (Wuarin et al., 1996; Li et al., 2002b). The eye is a special CNS sensory organ that is convenient to study and retinal cells undergo degeneration in diabetes (Barber et al., 1998; Kummer et al., 2003).

It has been proposed that a decline in insulin-like growth factor (IGF) activity contributes to diabetic neurological disturbances (Ishii, 1995). Circulating IGF-I levels are reduced in Type 1 and Type 2 diabetic patients (Tan and Baxter, 1986) and such reduction is greater in patients with neuropathy than without (Migdalis et al., 1995; Guo et al., 1999). IGF-I and IGF-II gene expression are reduced in the peripheral nerves and spinal cord, and IGF-II gene expression is reduced in the brain of STZ rats (Ishii et al.,

1994; Wuarin et al., 1994; Wuarin et al., 1996; Zhuang et al., 1997). IGF-II is the predominant IGF in the adult rat brain. The administration of low replacement doses of IGF-I or IGF-II prevents peripheral neuropathy in type 1 and type 2 diabetic rats independently of hyperglycemia (Ishii and Lupien, 1995; Zhuang et al., 1996; Zhuang et al., 1997; Schmidt et al., 1999). IGF-I can cross the B-CNS-B and a substantial fraction of IGF-I in the CNS is thought to arise from the circulation (Reinhardt and Bondy, 1994; Armstrong et al., 2000). Because circulating IGF-I levels are reduced in diabetic patients, diminished IGF activity may contribute to central neurological disturbances in diabetes.

To our knowledge, no treatment has been shown to prevent loss of neurons *in vivo* in the central nervous system in diabetes. It is of interest to determine whether IGF-I can prevent a loss of neurons in the CNS of diabetes. Since the methods for immunohistochemistry and cell counting in the neuroretinal layer have previously been optimized (Kummer et al., 2003) we chose to test whether subcutaneously infused IGF-I can prevent loss of neuroretinal cells in diabetic rats.

III. HYPOTHESES:

The purpose of this study was to test the following hypotheses: i) subcutaneous IGF-I administration can reduce apoptosis in retinal cells in diabetes, ii) systemically administered IGF-I is effective across the B-retinal-B, and iii) IGF can prevent retinal apoptosis in diabetes independently of unabated hyperglycemia.

IV. MATERIALS AND METHODS:

A. Animals: Rats used in this study are the same rats used for the cognition study in Chapter 2. Briefly rats were randomly assigned to one of three treatment groups. Two groups were rendered diabetic for 12 weeks and received either 20 μg/day/rat of IGF-I (Diab + IGF) or vehicle (Diab + vehicle) for the final 8 weeks of diabetes. Another group of rats was untreated (non-diabetic). After the last day of the Morris Water Maze visible platform test (12 weeks of diabetes), rats were weighed, blood was drawn for glucose measurements (Table 2-1) and euthanized. The eyes were dissected and placed in 4% paraformaldehyde. The fixed eyes were embedded in paraffin and 4 μm thin histological sections were made.

B. Immunohistochemistry: Paraffin-embedded retinal tissue sections were rehydrated through xylene and graded alcohols. Tissue sections were incubated in 0.25% Triton X-100 for five minutes. After a rinse in phosphate-buffered saline (PBS), sections were incubated for one hour with primary antibody. After rinsing 3 x 5 minutes in PBS, sections were incubated with a 1: 1500 dilution of biotinylated goat anti-rabbit or anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) for 60 minutes. Tissue sections were incubated for 20 minutes with horseradish peroxidase-conjugated avidin (Elite kit, Vector Laboratories). The sections were rinsed in 0.05 M Tris and antigens were detected with a diaminobenzidine (DAB) kit (Pierce); the brown/black reaction product was visualized by light microscopy. Negative controls consisted of incubations in 5% isotype control serum without primary antibody, and did not generate reaction product. After staining, immunoreactive cells were counted per 500 μm long retinal section for each rat. Sample labels were not visible to observers at the time of cell

counting. The figures show the means ± S.D. for numbers of immunoreactive cells per retinal section for each treatment group. Cell counts were analyzed with Tukey's multiple comparisons test. Differences between group means were accepted as significant at $P < 0.05$.

V. RESULTS:

A. Increased TUNEL Staining in Diabetic Rat Neuroretinal Cells: Effects of IGF-I: The TUNEL staining was used to measure fragmented DNA that is an indicator of apoptosis. An example of TUNEL-reactive cells in the diabetic rat is shown in Fig 4-1, panel B. The number of TUNEL positive cells increased approximately 6-fold in the photoreceptor layer ($P < 0.001$) and 8-fold in the inner nuclear layer (INL) ($P < 0.001$) in retinas from the (diab + veh) group versus (non-diabetic) group (Fig. 4-2). The number of TUNEL positive cells in the photoreceptor and INL retinal layers was significantly reduced ($P < 0.001$) in the (diab + IGF-I) group versus (diab + veh) group. TUNEL positive cells were increased approximately 2.5-fold in the ganglion cell layer (GCL) in the (diab + veh) group compared to the (non-diabetic) group, and IGF-I treatment reduced this mean value. However the changes in the GCL were not statistically significant due to smaller numbers of positive cells.

B. Increased Phospho-Akt Staining in Diabetic Rat Neuroretinal Cells: Effects of IGF-I: A photomicrograph shows phospho-Akt stained cells in the GCL and INL (arrows) (Fig 4-1, panel A). The number of cells positive for phospho-Akt was

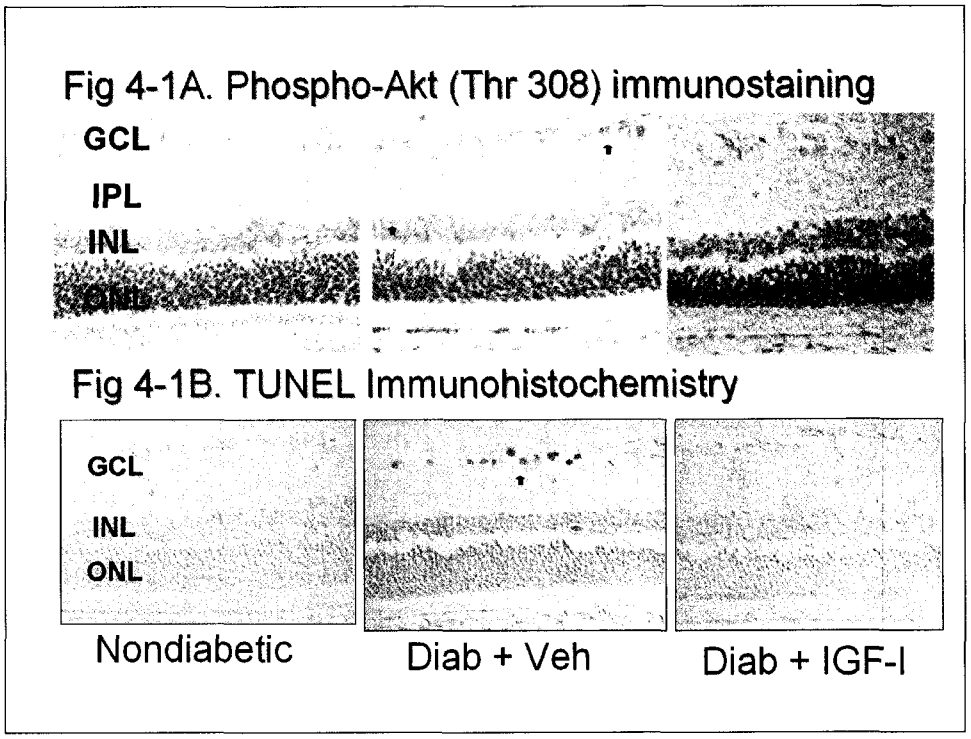

Fig. 4-1. Immunohistochemical localization of pro-apoptotic markers in retinas from diabetic rats, and effects of IGF-1 treatment. Rats were rendered diabetic by STZ for 12 weeks and then implanted subcutaneously with osmotic minipumps that released either 20 μg/day IGF-I (Diab + IGF-I) or vehicle (Diab + Vehicle) for the final 8 weeks. Sections of retinal tissue from nondiabetic control, (Diab + Veh), and (Diab + IGF) rats were stained with primary antibodies, as described in Methods. For each antibody, nondiabetic control tissue demonstrated little or no immunoreactivity for all markers. Panel A, phospho-Akt immunoreactivity showing positive cells (arrow) in the ganglion cell layer (GCL) and inner nuclear layer (INL) but not the inner plexiform layer (IPL), nor the outer nuclear for (Diab +Veh) retina. Panel B, TUNEL immunohistochemistry showing positive cells (arrow) in the GCL for (Diab +Veh) retina. Immunostaining was absent when primary antibody was omitted and replaced with isotype control serum.

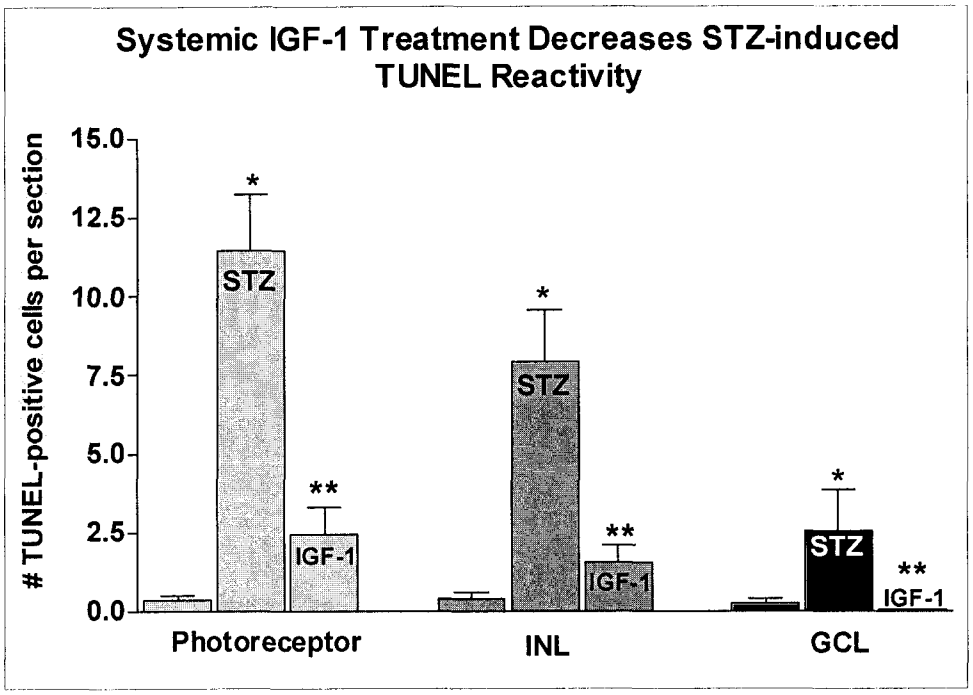

Fig. 4-2. The numbers of TUNEL-positive cells were increased in retina of diabetic rats, and such increase was prevented by IGF-I administration. Rats were rendered diabetic by STZ for 12 weeks and then implanted subcutaneously with osmotic minipumps that released either 20 μg/day IGF-I (STZ + IGF-I) or vehicle (STZ + Vehicle) for the final 8 weeks The mean numbers of TUNEL reactive cells per 500 μm-long retinal section were measured in the photoreceptor layer, GCL and INL for each group of rats. The mean numbers were significantly increased in (STZ + Veh) *vs.* nondiabetic control retina for all retinal layers ($P < 0.003$). This mean numbers were significantly reduced in (STZ +IGF) *vs.* (STZ + Veh) retinas ($P < 0.015$). The values are means ± S.D.

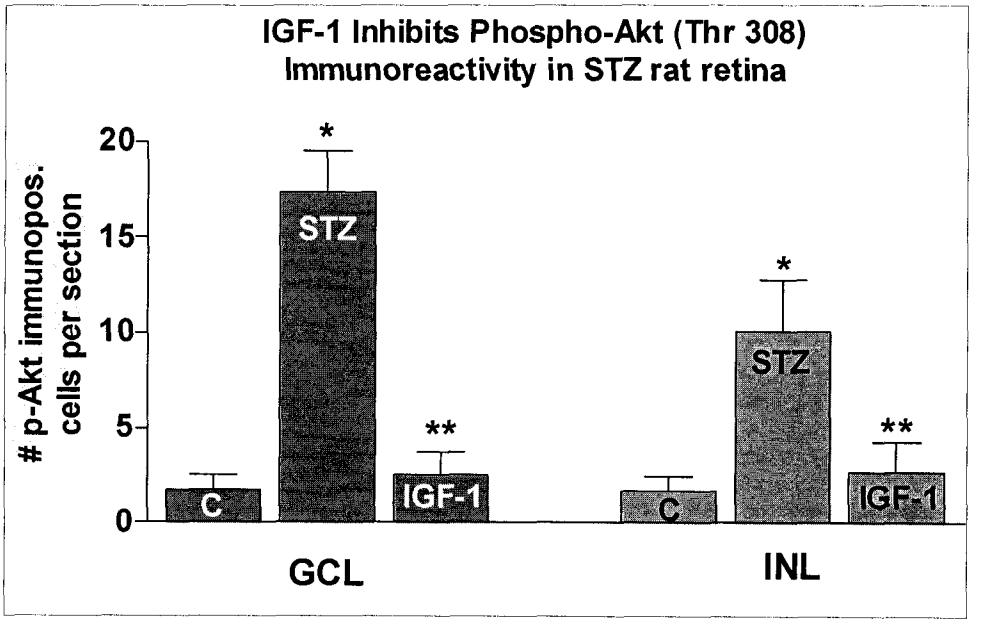

Fig. 4-3. The numbers of phospho-Akt positive cells were increased in retina of diabetic rats, and IGF-I administration reduced the number of such cells. The mean numbers of phospho-Akt positive cells per retinal section were measured in the GCL and INL. The mean positive cell count was significantly increased in (STZ + Veh) *vs.* nondiabetic control retina for the GCL ($P < 0.0001$) and the INL ($P < 0.015$). The mean cell count was significantly reduced in (STZ + IGF) *vs.* (STZ + Veh) retina ($P < 0.015$). The values are means $\pm$ S.D.

increased 8-fold in the GCL ($P < 0.001$) and 3-fold in the INL ($P < 0.01$) in (diab + veh) vs. (non-diabetic) rats (Fig. 4-3). Systemic IGF-1 treatment significantly ($P < 0.05$) reduced the number of phospho-Akt positive cells in both the GCL and INL in the (diab + IGF-I) group versus the (diab + veh) group to approximately the level observed in nondiabetic rats.

VI. DISCUSSION:

A. Neurotretinal Cell Loss in Diabetes Suggests that Cell Loss Is associated with Brain Atrophy: These data show that phospho-Akt as well as TUNEL immunostaining are increased in retinal cells in diabetic rats. The TUNEL method detects free 3'-OH ends on DNA, and apoptotic cell death results in DNA fragmentation and increased numbers of 3'-OH ends (Wyllie et al., 1984; Gavrieli et al., 1992). The increased TUNEL immunoreactivity observed here in retinal cells of diabetic rats indicates that apoptosis is increased in diabetes. Apoptosis also occurs in regions of the brain other than the retina. Positive TUNEL staining is observed in the hippocampus of Type 1 diabetic rats (Li et al., 2002b). It is likely that cell loss slowly contributes to brain atrophy in diabetes. In fact, there is no reduction in brain size after two weeks of diabetes in rats (Wuarin et al., 1996), and apoptosis was not absent at two weeks. Neurogenesis occurs in the hippocampus to continuously regenerate neurons (Cameron et al., 1993; Cameron and McKay, 2001). Neurogenesis could counter the loss of brain cells in this region. However, neurogenesis in the hippocampus of diabetic rats is reduced (Jackson-Guilford et al., 2000). Over time, atrophy of the brain in diabetes probably occurs due to

multiple events that include increased loss of cells, reduced neurogenesis, reduced cell size and reduced levels of protein synthesis and/or stability.

B. IGF-I Administration Prevents Apoptosis in Neuroretinal Cells in Diabetes: These data show that IGF-I administration prevented both the increase in phospho-Akt as well as TUNEL immunostaining in retinal cells in diabetic rats. Whatever the mechanism, subcutaneous IGF-I administration prevented neuroretinal cell death in diabetic rats. These are the first data to show, to our knowledge, that IGF can prevent cell death in the nervous system in the context of diabetes. Because IGF administration prevented cell death in rats diabetic for more than 11 weeks, cell death is possibly not due to hyperglycemia nor its secondary consequences per se. Alternatively, the decrease in neurotrophic IGF levels in diabetes may be a more substantial risk factor for neuron loss. IGF-I and IGF-II can prevent neuron loss *in vitro* (Recio-Pinto et al., 1986; Aizenman and de Vellis, 1987) and *in vivo* (Neff et al., 1993; Fernandez et al., 1998; Pu et al., 1999), and anti-IGF antiserum can increase neuron death in non-diabetic animals (Pu et al., 1999). Anti-IGF antiserum can also inhibit learning/memory as shown in Chapter 2. Cell loss in the nervous system is additionally observed in IGF-I knock-out mice (Beck et al., 1995).

IGF-I may prevent reduced neurogenesis as well as cell loss in diabetes. Hippocampal neurogenesis is increased by IGF-I in hypophysectomized rats that received IGF-I treatment (Aberg et al., 2000) and transgenic mice that over expressed IGF-I (O'Kusky et al., 2000). Exercise-induced increases in hippocampal neurogenesis are mediated by circulating IGF-I (Trejo et al., 2001). IGF's may reduce the loss of cells and

possibly prevent reduced neurogenesis in the brain of diabetes partially but incompletely preventing brain atrophy. It is unresolved whether IGF-I treatment initiated earlier than four weeks after induction of diabetes might prevent brain atrophy.

C: Hyperglycemia: IGF-I administration effectively prevented retinal apoptosis in diabetic rats despite unabated hyperglycemia. IGF-I treatment had no effect on severity of hyperglycemia or reduced body weight in diabetic rats (Table 2-1). Body weight is a sensitive index of poor metabolic control. IGF-I or IGF-II administration, despite unabated hyperglycemia in experimental diabetes, can prevent or reverse various neurological disturbances including impaired sciatic nerve regeneration (Ishii and Lupien, 1995; Zhuang et al., 1996), hyperalgesia (Zhuang et al., 1996; Zhuang et al., 1997), neuraxonal dystrophy (Schmidt et al., 1999), abnormal gene expression in brain (Armstrong et al., 2000), and impaired learning/memory (Lupien et al., 2003). These observations together are of clinical interest, because they add to the growing body of evidence supporting the hypothesis that IGF administration can prevent peripheral diabetic neuropathy as well as central neurological disturbances even in poorly controlled diabetic patients.

VI. ACKNOWLEDGMENTS:

This work was a collaborative project with Gail Seigel who performed all the immunohistochemistry and analysis. This work was supported by CDC Grant R49/CR811509 and NIDDKD Grant RO1 DK53922. S.B.L. is a predoctoral fellow supported in part by NINDS Training Grant 1T32NS43115. The authors thank Janet

Wagner for assisting with histological specimens.

CHAPTER 5

Effect of Anti-IGF Antiserum on Learning and Memory

The experiments in this chapter combined with the experiments in chapter 2 will be published in the *Journal of Neuroscience Research,* 74:512-523 (2003).

Effect of Anti-IGF Antiserum on Learning and Memory

Sean B. Lupien and Douglas N. Ishii

I. ABSTRACT: Insulin-like growth factor II (IGF-II) is a neurotrophic factor expressed at the highest levels in the brain, spinal cord, and nerves of adult mammals, suggesting a selective role for the nervous system. IGF-I and IGF-II are found in cerebrospinal fluid. The role of IGFs in the adult mammalian brain is unclear. Synapse formation, neurogenesis, and protein synthesis are events associated with learning and memory. Because IGFs are involved in synapse formation and axon elongation in peripheral nerves as well as mitosis in neuroblasts, we tested the hypothesis that brain IGF is essential for learning and memory. A cannula, connected to a miniosmotic pump, was implanted into the left lateral ventricle of adult rats. The pump contained IGF-II antibody (IGF-II Ab) or vehicle (pre-immune serum). IGF-II Ab binds to IGFs and therefore may inhibit activity. Rats were subjected to a passive avoidance test of their ability to learn and remember not to instinctively enter a dark chamber after 10 days of infusion. Rats were habituated to pass from a light to a dark chamber for 2 days. On day 3, an electric shock was administered on entry into the dark chamber. On days 4-6, the latency to enter the dark chamber tested learning and memory (avoidance of shock). The rats were given a reinforcement shock upon entry. The mean latency for all groups on days 1-3 during habituation was less than 15 seconds with no significant differences. On days 4, 5, and 6, the mean latencies of the IGF-II Ab group were significantly shorter than that of the pre-

immune serum group ($P < 0.04$, 0.02, 0.004, respectively). These data show that endogenous IGF in cerebrospinal fluid regulates or supports learning and memory. Learning and memory deficiencies are observed in aging humans and rodents, and IGF-II gene expression is reduced in the brain of aged rats.

II. INTRODUCTION:

The experiments in Chapter 2 demonstrate that systemic infusion of insulin-like growth factor-I can prevent cognitive dysfunction in diabetic rats. It is of interest to determine whether endogenous IGFs in the brain are essential for learning and memory.

Insulin-like growth factors (IGF-I and IGF-II) are neurotrophic factors. IGF-II is expressed at the highest levels in the brain, spinal cord, and nerves of adult rats, suggesting an important role in the nervous system. Most is known about the role of IGFs in injury, such as regeneration or repair of the PNS and CNS (Ishii and Pu, 1999) (D'Ercole et al., 1996). IGF-I and IGF-II knockout mice have smaller in brains (DeChiara et al., 1990; Liu et al., 1993) and IGF-I transgenic mice have increased brain size (Carson et al., 1993). However, the normal role of IGF in the CNS of intact adult mammals is unclear.

Learning and memory may be dependent at least in part on synapse formation. Because IGFs induce sprouting at nerve terminals (Caroni and Grandes, 1990) and IGF gene expression is correlated with synapse formation, IGFs may potentially support learning and memory. Brain IGF-II gene expression is reduced in aged or diabetic rats in association with reduced cognitive function. Infusion of IGF-I into the ventricles

ameliorates learning deficits in aged rats (Markowska et al., 1998). In Chapter 2, I showed that subcutaneous IGF-I administration prevented cognitive impairments in diabetic rats. However, it is still not known whether endogenous brain IGF normally supports cognitive processes.

III. HYPOTHESIS:

The purpose of this study was to test the hypothesis that endogenous IGFs in the brain are essential for learning and memory.

IV. MATERIALS AND METHODS:

A. Anti-IGF-II Antiserum and Intracerebroventricular Infusion of Anti-IGF-II Antiserum: An anti-IGF-II antiserum was prepared by Dr. Donald J. Marsh of our group. Human IGF-II was coupled to keyhole limpet hemocyanin and New Zealand rabbits were immunized (complete Freund's adjuvant) after withdrawing preimmune serum. The anti-IGF-II antiserum (1:1000) was tested on a Western blot following electrophoresis of 40 ng protein per lane in a 15% polyacrylamide gel containing sodium dodecylsulfate. Human and rat IGF-II were detected using Lumi-Phos 530 chemiluminescence substrate (Boehringer Mannheim, IN), and bands were of the expected size and mobility. By contrast, human IGF-I, human proinsulin and porcine relaxin were not detected. Preabsorption of the antibody with excess IGF-II neutralized its capacity to detect IGF-II on Western blots. However, anti-IGF-II antiserum diluted less (1:250) did detect IGF-I, showing that lower affinity IGF antibodies were present.

Harlan Sprague-Dawley rats (12-week old males) were anaesthetized with ketamine (110 mg/kg) and xylazine (8 mg/kg) and placed in a stereotaxic apparatus. Following the coordinates of Paxinos and Watson (1986) a hole was drilled through the skull at -0.9 mm caudal and left 1.5 mm from bregma. A cannula was inserted 4.0 mm deep into the left lateral ventricle using the Alzet Brain Infusion Kit (Durect Corp., Cupertino, CA). The cannula was attached to tubing routed to an osmotic minipump that was implanted s.c. between the shoulder blades. Rats were randomly assigned to treatment groups that received 12 µl per day i.c.v. of either 40% anti-IGF-II antiserum (IGF Ab) or 40% pre-immune serum (PIS) diluted in artificial cerebrospinal fluid: 10 mM NaCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM K_2HPO_4, 10 mM glucose, pH 7.4. After 2 weeks of treatment, rats were subjected to the Passive Avoidance Test. At the end of the experiment, rats were decapitated and 20 µl of bromophenol blue was gently injected through the catheter. The presence of bromophenol blue in the lateral ventricles was used to confirm correct cannula placement.

B. Passive Avoidance: A wooden box had two 13.5" wide 12" high x 15" deep chambers separated by a wood partition with a 5" x 3.5" sliding door (Fig 5-1). One chamber was white and illuminated by an overhanging lamp through a clear plexiglass lid, whereas the other chamber was black and covered by a wooden lid. Metal grids were placed on the floor of both chambers, but only the grid in the dark chamber was attached to a constant current shock device. A Constant Current Shocker Model 58006 and Grid Scrambler Model 82500 (Lafeyette Instrument Co., Lafeyette IN) were connected to the grid.

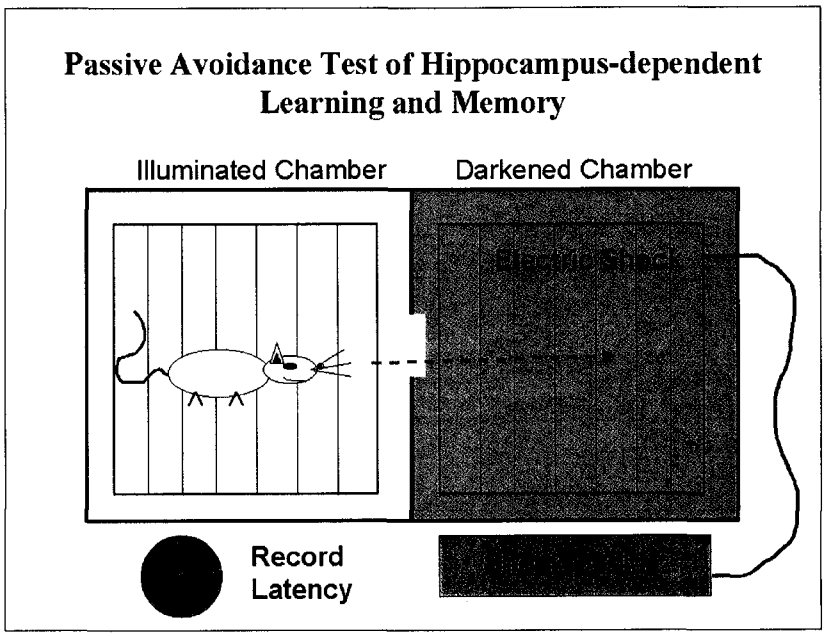

Fig. 5-1. Passive avoidance learning and memory test. For each of two days, rats were habituated by being placed in a light chamber and the latency time to instinctively pass through a door into a dark chamber was recorded. On the third day, a metal grid on the floor of the dark chamber administered five successive electric shocks of 0.3 mA to the rats. Over days 4, 5 and 6, learning and remembering to avoid a shock was measured by the latency of the rats to enter the dark chamber (maximum 300 seconds). The rats were given a reinforcement shock if they entered.

Ten days postsurgery, rats were subjected to a 6 day passive avoidance test of their ability to learn and remember to avoid instinctively entering the dark chamber. Each day prior to a trial, the rats were relaxed by gentle handling for 2-5 minutes. Rats were placed in the light chamber and allowed to adjust to the environment for 30 sec before opening the sliding door to the dark chamber. For the first two days, rats were habituated by placing them into the light chamber and recording their latency to instinctively enter the dark chamber. On the third day, when the rats entered the dark chamber, five successive electric shocks (0.3 mAmp, 400 msec) were administered. On Days 4, 5 and 6, learning and remembering to avoid a shock was measured by their latency to enter the dark chamber (maximum 300 seconds). The rats were given a reinforcement shock if they entered.

C. Statistical Analysis: The Mann-Whitney U-test for non-parametric comparisons was used to analyze the passive avoidance test results.

V. RESULTS:

Effect of Anti-IGF-II Antiserum on Learning and Memory in a Passive Avoidance Test: It was of interest to test whether a decline in IGF-I activity could produce cognitive deficits in the absence of hyperglycemia and severe metabolic disturbances. It was further desired to test whether IGF in brain was normally essential for hippocampal-dependent learning and memory. An anti-IGF-II antiserum (IGF-Ab) or pre-immune serum (PIS) was constantly infused into the left lateral ventricle of adult nondiabetic rats. After 10 days, learning/memory was tested using a passive avoidance

device (Fig. 5-1). Rats were placed into a well-lit chamber containing a trap door entrance into a dark chamber. By instinct, both groups of rats entered the dark chamber in less than 8 sec $\pm$ 8 sec during the 2 days of habituation as well as the day of the first shock. On the third day, all rats received a mild electrical shock when they entered the dark chamber. The learning/memory test involves their remembering (latency) to avoid entering the dark chamber. Should they forget and enter the dark chamber, a reinforcement shock was given.

The latencies of rats receiving pre-immune serum immediately increased on Test Day 1 (Fig. 5-2), and remained elevated throughout the three days of testing. By contrast, the latencies of rats treated with the anti-IGF antiserum were significantly reduced on Test Days 1, 2 and 3 vs. rats treated with the preimmune serum ($P < 0.04$, $P < 0.02$, $P < 0.004$, respectively).

VI. DISCUSSION:

An Anti-IGF Antiserum Inhibited Learning and Memory: The present study shows that infusion of an anti-IGF antiserum into the lateral ventricles inhibits learning/memory in nondiabetic rats in a passive-avoidance test (Fig. 5-2). Infusion of IGF binding protein-6, also inhibits learning/memory in this test (Lupien et al., 2000). Hippocampal lesions prevent passive-avoidance learning (Cogan and Reeves, 1979). Therefore, brain IGF is essential for learning/memory in this hippocampus-based test. This suggests that the support of learning/memory is among the neurobiological roles served by IGFs. In fact, mental retardation is observed in a patient with a mutation in the

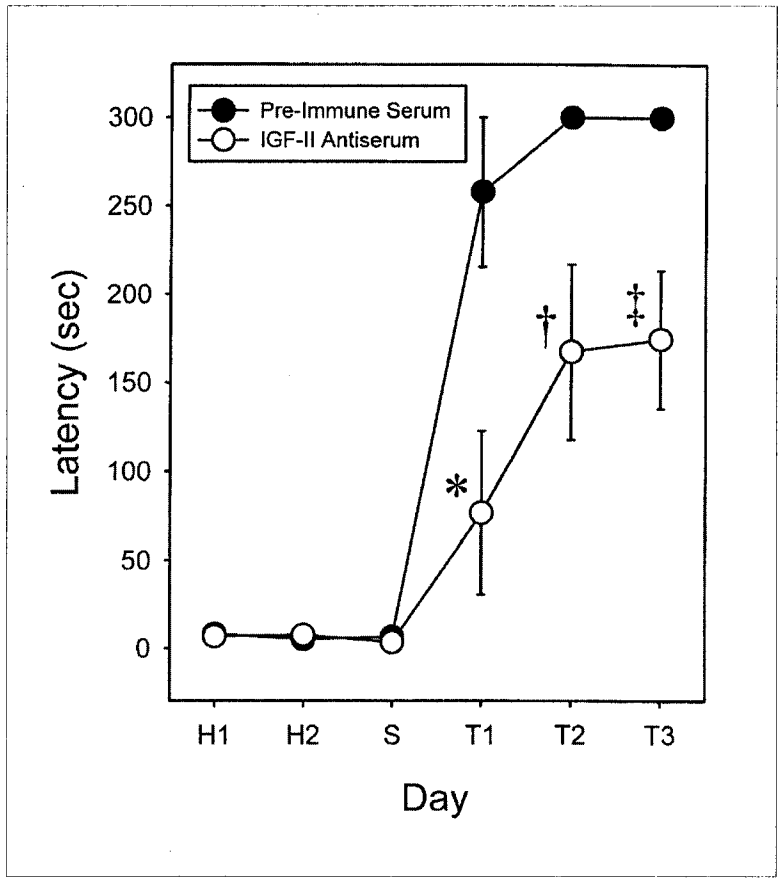

Fig. 5-2. An anti-IGF-II antiserum prevented the acquisition of learning and memory in a passive avoidance test. Nondiabetic adult rats were randomly assigned to treatment groups. Cannulas were implanted into the brain lateral ventricles connected to osmotic minipumps (2 weeks duration) that released either 4.8 µl/day anti-IGF-II antiserum (N = 6 rats) or a comparable volume of preimmune serum (N = 6). After 10 days, rats were subjected to a passive avoidance test consisting of two days of habituation (H) to instinctively enter a dark chamber. On the third day rats entering the dark chamber received a shock (S). On three subsequent test days (T) the latencies for rats to forget the shock and enter the dark chamber was measured. The mean latencies were significantly shorter in the IGF-II antiserum *vs.* pre-immune serum treated groups on Test Days 1, 2 and 3 (*P = 0.04, †P = 0.02, and ‡P = 0.004, respectively). Data were analyzed using the Mann-Whitney U test. Values are means ± SEM. SEM smaller than symbols are not shown.

IGF-I gene (Woods et al., 1997), and low circulating IGF-I levels are correlated with poorer cognitive performance in elderly human subjects (Aleman et al., 1999). A quantitative trait locus linked to general cognitive ability has been mapped to the IGF type 2 receptor (Chorney et al., 1998). These data together are consistent with a model in which circulating IGFs helps to maintain central nervous system functions, and a loss of IGF activity may confer increased risk for cognitive decline in diabetes and progression to dementia.

Because both IGF-I and IGF-II levels are reduced in diabetes, it remains uncertain whether the cognitive disturbances arise because of loss of IGF-I, IGF-II, or both. Peripheral neuropathy can be prevented in diabetic rats by administration of either IGF-I or IGF-II. Because both IGF-I and IGF-II activate the type 1 IGF receptor, what may be key is that overall IGF signaling is reduced in diabetes. The anti-IGF antiserum was raised against IGF-II, and at 1:1000 dilution did not recognize IGF-I on western blots. However, at higher concentrations the antiserum might bind to IGF-I, and higher concentrations (1:2.5 dilution in pumps) are present at and near the i.c.v. release site. Consequently, it is possible that this antibody bound IGF-I as well as IGF-II. It is possible that in order to block learning/memory it may be necessary to block the actions of both IGF-I and IGF-II to prevent activation of the type 1 IGF receptor. Also, perhaps minimal stimulation of the type 1 IGF receptor by IGF supports learning/memory, and antiserum sequestration of IGF-I, IGF-II or both may remove such stimulation. No attempt was made at this time to assess the relative contribution of IGF-I and IGF-II.

It would be interesting to determine whether learning and memory is impaired in

diabetic rats in the passive avoidance as well as Morris water maze test. However diabetic rats have hyperalgesia (increased sensitivity to pain)and would have a greater response to a shock stimulus. Consequently, they cannot be readily compared to nondiabetic rats in the passive avoidance test.

VII. ACKNOWLEDGMENTS:

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CHAPTER 6

Generation of Transgenic Mice Containing an Insulin-like Growth Factor-II cDNA to Study the Effects of IGF-II Over Expression in the CNS

Generation of Transgenic Mice Containing an Insulin-like Growth Factor-II cDNA to Study the Effects of IGF-II Over Expression in the CNS

Sean B. Lupien and Douglas N. Ishii

I. ABSTRACT: Insulin-like growth factor II (IGF-II) is a neurotrophic factor expressed at the highest levels in the brain, spinal cord, and nerves of adult mammals, suggesting a selective role for the nervous system. To study the effects of IGF-II in the CNS, we developed an IGF-II transgenic mouse designed specifically to over express IGF-II in the CNS. We generated an IGF-II transgene that contains an IGF-II cDNA downstream from the Glial Fibrillary Acidic Protein (GFAP) promoter and upstream from an SV40 Poly(A) sequence. The IGF-II transgene was microinjected into the male pronucleus of one-cell stage mouse embryos and then transplanted into pseudopregnant female mice. Four founder mice containing the transgene were identified by PCR and then bred to non-transgenic mice to produce offspring. RNA was purified from the brains of transgenic and non-transgenic litter mates and probed for IGF-II mRNA on a Northern blot to determine whether the transgene was expressed. A band was present near the size of the endogenous IGF-II mRNA but no band was present near the expected size of the IGF-II transgene. Since there was no IGF-II transgene expression and no apparent phenotypic changes, such as brain size or body weight, in transgenic mice versus non-transgenic litter mates, the project was considered an unsuccessful attempt to over express IGF-II specifically in the CNS of mice. Other unsuccessful attempts to over-express IGF-II in

mice have been reported. Since the IGF-II gene is parentaerly imprinted, it is possible that an IGF-II transgene may be susceptible to inactivation.

II. INTRODUCTION:

Regulation of brain size in animals is not understood. Evidence suggests that IGF-I and IGF-II may have roles in the development of the nervous system and regulation of overall brain size. Studies that have generated transgenic mice over expressing IGF-II have found an in increase in embryonic and postnatal body size as well as disproportionate increases in the size of various organs such as kidneys, heart, and liver (Rogler et al., 1994; Ward et al., 1994; Wolf et al., 1994; Buul-Offers et al., 1995; Sun et al., 1997). Increases in IGF-II mRNA were shown to correlate to increased size of embryos and the respective postnatal organs. However, none of these studies have reported a disproportionate increase in brain size. This may be because brain IGF-II mRNA has not been reported to increase in any line of IGF-II transgenic mice. Adult mice that over express IGF-I have increased body weights by 25-85% of normal depending on the line of mice. However, body weight increase was not detected until 6-8 weeks of age (Mathews et al., 1988). An increase in brain size of IGF-I transgenic mice was detected after approximately postnatal day 20 (P20). By P55, brain weight increased 55% over normal and IGF-I mRNA in the brain was approximately 2-fold higher (Carson et al., 1993). Over expression of IGF-II by introducing an IGF-II receptor null mutation and an H19 null mutation results in 100% mortality. H19 is a gene located near the IGF-II receptor locus that regulates IGF-II receptor gene expression. Live mutants at E16.5

and E17.5 (20 day gestation) were 200% larger than normal siblings which was reflected in weights of various organs but not always proportionately (Eggenschwiler et al., 1997).

The highest level of IGF-II mRNA are found in the brain, spinal cord, and nerves among tissues in adult rats suggesting a selective role for the nervous system. No report has described the effect of increased IGF-II in the CNS. It is of interest to generate transgenic mice that over express IGF-II specifically in the CNS and determine whether increased IGF-II can increase brain size and if so, by what mechanisms. Also, diabetic transgenic mice that constituitively over-express IGF-II may be resistant to learning and memory impairments.

To over-express IGF-II specifically in the CNS, we cloned an IGF-II cDNA downstream from the astrocyte specific promoter, Glial Fibrillary Acidic Protein. IGF-II is a soluble protein that when released from cells can have autocrine and paracrine activity. Since about 40% of the cells in the brain are astrocytes, we thought that over-expression of IGF-II in these cells would greatly increase the levels of IGF-II in the CNS. We used the 3-prime end of the GFAP promoter that was shown to express luciferase reporter activity in cultured rat astrocytes but not in fibroblasts (Miura et al., 1990). It was important to localize IGF-II transgene expression specifically in the CNS because expression in other tissues could alter function that might indirectly cause changes in the CNS.

III. HYPOTHESES:

The purpose of this study was to test the hypotheses that i) IGF-II is associated

with brain development, and ii) IGF-II regulates brain size.

IV. MATERIALS AND METHODS:

A. IGF-II Transgene Construction: The rat IGF-II cDNA clone 27 in pUC9 containing an inverted 5' UTR and 3' UTR and the entire prepro IGF-II coding sequence was a gift from Argiris Efstratiadis. The Glial Fibrillary Acidic Protein (GFAP) promoter was cloned from mouse genomic DNA by PCR. Primers were constructed to amplify the GFAP promoter from region -256 bases to +12 bases that includes the transcription start site at +1 base. The GFAP promoter was cloned into a pGL3 vector (Promega) and transformed into DH5alpha bacterial cells (Invitrogen). The plasmid was purified from a bacterial culture using a Qiagen kit. IGF-II cDNA was cloned into the pGL3 vector downstream of the GFAP promoter and subsequently transformed into DH5alpha cells. The plasmid was purified from a bacterial culture using a Qiagen kit. The IGF-II transgene containing the GFAP promoter, IGF-II cDNA and SV40 poly(A) sequence was isolated from the pGL3 plasmid by restriction enzymes and agarose gel purified using a Qiagen kit. The structure of the IGF-II transgene construct is shown in Fig. 6-1.

B. Generation of Transgenic Mice: The linearized IGF-II transgene was microinjected into the male pronucleus of one-stage cell FVB mouse embryos (performed by Michele Simms in the Biomedical Sciences Transgenic Animal Facility). The embryos were transplanted into pseudo-pregnant FVB mice.

C. Assay for Identification of Transgenic Mice: Oligonucleotide primers were synthesized to PCR amplify a region of the IGF-II transgene containing the GFAP

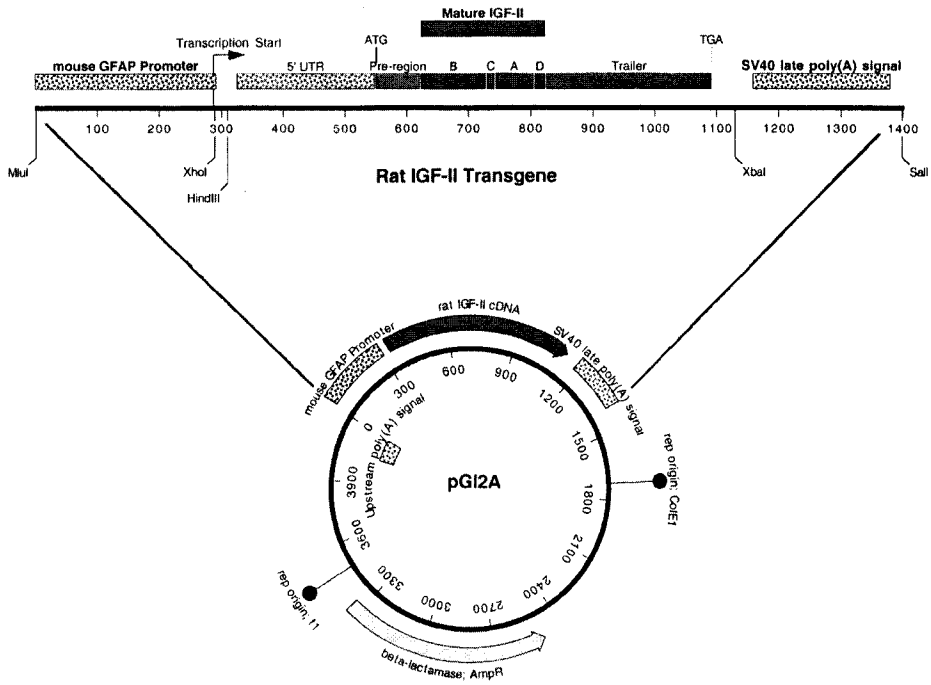

Fig. 6-1. Construct of the rat IGF-II transgene used for microinjection into male pronuclei of fertilized mouse eggs. The mouse glial fibrillary acidic protein (GFAP) promoter (256 bp of the 3-prime end) was cloned into the pGL3-basic vector upstream of the SV40 late poly(A) sequence. Rat IGF-II cDNA was inserted between the GFAP promoter and the SV40 late poly(A) sequence. Prior to microinjection, the rat IGF-II transgene was excised from the vector and the DNA was gel purified.

promoter and the IGF-II cDNA. Tails were snipped from mouse pups and digested in proteinase K. Genomic DNA was purified by extraction in chloroform and isoamyl alcohol. The presence of transgene was determined by PCR amplification of genomic DNA.

After the assay was developed, it was found that the IGF-II cDNA was cloned into the pGL3 plasmid in the inverted orientation. Although the IGF-II cDNA was sequenced prior to cloning, the inverted orientation occurred when the IGF-II cDNA was cloned into pBluescript at an ECOR1 site. This intermediate cloning step was performed so as to use the multiple cloning site of pBluescript to direct orientation into the pGL3 plasmid by isolating the IGF-II cDNA with BamH1 and XbaI restriction sites. The multiple cloning region of pBluescript can be in the plus or minus direction and I was inadvertently advised of the incorrect orientation. After realizing this, the IGF-II cDNA was re-cloned into pGL3 in the correct orientation and this was confirmed by DNA sequencing.

D. Assay for Transgene Expression in Transgenic Mice:

1. RNA Purification: RNA from the brains of 6 week-old transgenic and non-transgenic mice was isolated by the guanidinium thiocyanate procedure of Chomczynski and Sacchi (1987). RNA concentration and purity were estimated by absorbance at 260 and 280 nm.

2. Northern Blot: Forty μg of total RNA was electrophoresed through a 1.5% agarose gel containing 10% formaldehyde, as described (Lehrach et., 1977). Ethidium bromide staining determined the position of 18S and 28S rRNAs and confirmed that roughly equivalent amounts of undegraded RNA had been loaded. The RNA was

transferred to a nitrocellulose membrane, cross-linked to RNA using UV light at 0.12 J/cm^2 (Stratagene) then baked at 80°C for 1 hour under vacuum.

3. **Preparation of an IGF-II cDNA Probe:** Rat IGF-II cDNA clone 27 in pUC9 plasmid was isolated from bacterial culture using a Qiagen kit. Purified plasmid DNA along with primer pairs flanking the IGF-II cDNA were used to PCR amplify the IGF-II cDNA. The PCR product was run on a 1% agarose gel and purified with a Qiagen kit. The gel purified IGF-II cDNA, alpha-^{32}P-dCTP (Amersham), T7 primer, and dNTPs were used in primer extension with Taq DNA polymerase (Promega) to produce a single-stranded anti-sense cDNA probe and subjected to 40 cycles of thermocycling (95°C for 1 min, 50°C for 2 min, and 72°C for 3 min) in a Perkin Elmer 480 Thermo Cycler followed by a 10 min final extension at 72°C and a hold at 4°C. The probe was purified to remove unincorporated nucleotides in a Bio-Spin P-30 Chromatography column (Bio-Rad).

4. **Hybridization to RNA:** The nitrocellulose blot was hybridized to ^{32}P-labeled IGF-II cDNA probe for 16 hours at 42°C. Hybridization buffer contained 6X SSPE (1X SSPE is 150 mM NaCl, 10 mM NaH2PO4, and 1 mM EDTA, pH 7.4), 5X Denhardt's solution, 0.5% SDS, 100 μg/ml salmon sperm DNA, and 50% formamide. The blot was washed using a procedure to detect low abundance transcripts (Sambrook et al., 2000). The blot was subsequently exposed to a phosphorscreen, and scanned on a PhophorImager scanner (Molecular Dynamics, Sunnyvale, CA).

V. RESULTS:

A. **IGF-II Transgene Incorporated Into Four Mice:** Mice were screened for

incorporation of the transgene by PCR using primers designed to amplify a region of the transgene consisting of the GFAP promoter and the IGF-II cDNA. By this screening method, the IGF-II transgene was successfully incorporated into the genome of four mice (Fig. 6-2). These four founder mice were bred to non-transgenic mice to produce four lines of transgenic mice: line 42, line 68, line 70, and line 77.

B. Assay for Expression of the IGF-II Transgene: Transgenic and non-transgenic litter mates were weighed and the brain was dissected and weighed. There was no difference in body weight or wet brain weight in any of the transgenic mouse lines versus non-transgenic mice. RNA was purified from the brains of transgenic and non-transgenic litter mates from lines 42, 70 and 77. The RNA was assayed for IGF-II gene expression by Northern analysis. The data showed a band near the expected size of the endogenous IGF-II transcript (4.7 Kilobases) (Fig. 6-3). There was no band near the expected size of the IGF-II transgene transcript (1.0 Kilobases) in neither the transgenic nor the non-transgenic mice.

VI. DISCUSSION:

IGF-II Transgene Does Not Produce Transcripts in Transgenic Mice: The data show that the IGF-II transgene was successfully incorporated into the genome of four founder mice (Fig. 6-2). However, IGF-II transgene expression was not detected in any of the transgenic lines assayed (Fig. 6-3). Body weight and brain weight were compared anyway in transgenic versus non-transgenic litter mates and no differences were found. There are many possible reasons why the IGF-II transgene did not produce transcripts.

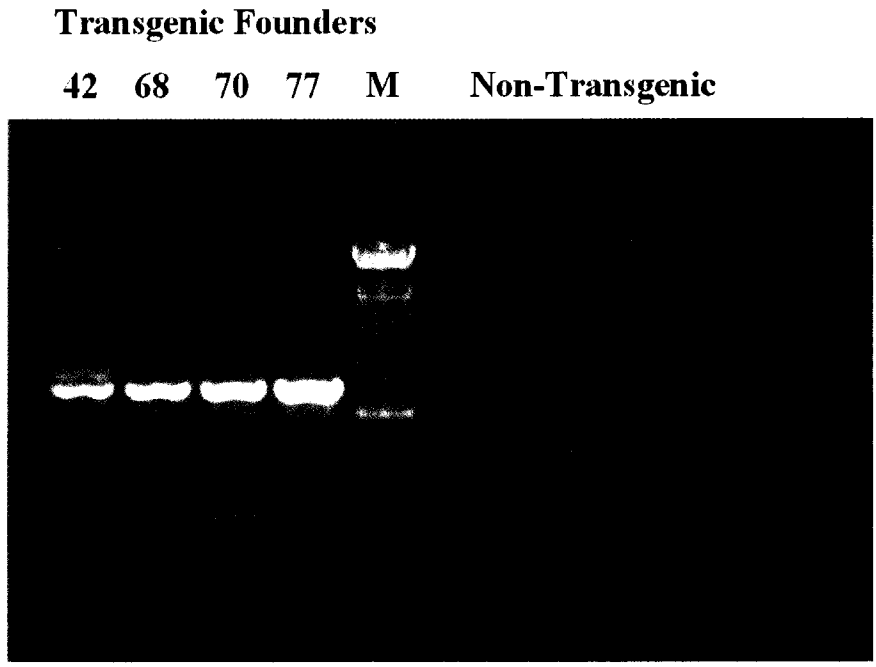

Fig. 6-2. Four transgenic founder mice were identified by PCR. Tips of the tails of mouse pups were digested in proteinase K and genomic DNA was purified. Presence of the IGF-II transgene was determined by PCR amplification of a region specific to the transgene. Four mice were found to contain the IGF-II transgene (42, 68, 70, and 77). Shown here are the PCR products electrophoresed on a 1% agarose/Ethidium Bromide gel from the four founder mice and four non-transgenic littermates near the expected size of the PCR product. M is a 100 base-pair marker.

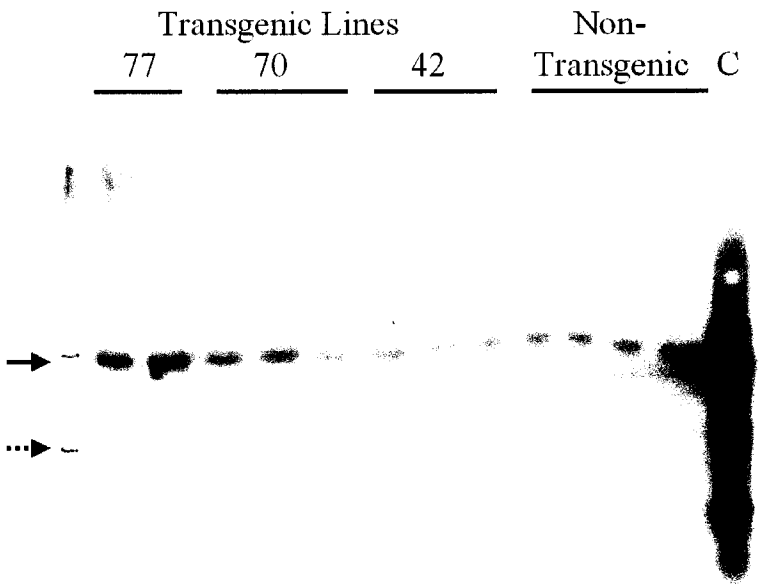

Fig. 6-3. IGF-II transgene expression was not detected in transgenic mice. Total RNA was purified from brains of transgenic and non-transgenic littermates. RNA was electrophoresed on a 1.5% agarose/formaldehyde gel, transferred to an immobilization membrane, and hybridized to a rat IGF-II cDNA probe. Mice from three transgenic lines (42, 70, and 77) and four non-transgenic littermates showed a band near the expected size of the endogenous IGF-II transcript (4.7 Kilobases) (solid arrow) but no band was present near the expected size of the IGF-II transgene transcript (1.0 Kilobases) (dashed arrow) in neither the transgenic nor the non-transgenic mice. C is a positive control for IGF-II detection that is total RNA from postnatal day 5 rat gastrocnemius muscle.

The endogenous IGF-II gene as well as the IGF Type 2 receptor are parentally imprinted in the brain (DeChiara et al., 1991). For the IGF-II gene and the IGF Type 2 receptor only one allele is expressed and the other is silenced. It is possible that the IGF-II transgene is susceptible to the same gene silencing factors. Other unsuccessful attempts to over-express IGF-II in mice by DNA microinjection in one-cell stage embryos have been reported (Palmiter and Brinster, 1986). Another possible reason for unsuccessful transgene transcription may be due to problems inherent to the transgene itself. The truncated 3-prime end of the GFAP promoter expressed luciferase reporter activity in cultured rat astrocytes but not in fibroblasts (Miura et al., 1990) and this was used for our transgene construct. However, the truncated GFAP promoter may require additional up-stream sequences for expression *in vivo*. The rat IGF-II cDNA has an inverted repeat in the 5-prime untranslated-region that may be required for transcription. It is also possible that all sites where the transgene incorporated into the mouse genome are not suitable for transcription to occur.

It remains of interest to study the effects of IGF-II over expression specifically in the CNS. The method used here successfully produced transgenic mice but was unsuccessful to accomplish over expression of IGF-II in the CNS. Therefore, no data was accumulated in regard to addressing the hypotheses on the effect of IGF-II over expression on CNS development.

VII. ACKNOWLEDGMENTS: I would like to thank Dr. Richard Bowen for providing instruction and direction for constructing the IGF-II transgene and subsequent

generation of transgenic mice. I would also like to thank Michelle Simms for performing the IGF-II transgene microinjections into one-cell stage fertilized mice, the subsequent implantation into pseudopregnant mice and assistance in identifying and maintaining the transgenic mouse colony.

CHAPTER 7

Summary of Results, Conclusions, Significance and Suggestions for Future Experiments

I. SUMMARY OF RESULTS:

1. Subcutaneous IGF-I administration to rats diabetic for 12 weeks significantly ($P<0.03$) prevented impaired cognitive ability in the Morris Water Maze submerged platform test.
2. Subcutaneous IGF-I administration had no effect on hyperglycemia, body weights, brain wet weights and hippocampi wet weights in diabetic rats.
3. Protein per brain and protein per wet brain weight were reduced in diabetes and IGF-I administration partially prevented reduced protein per brain and completely prevented reduced protein per wet brain weight in diabetes.
4. Poly(A)+ RNA per brain was reduced in diabetes.
5. 18S ribosomal RNA per brain and per wet brain weight were reduced in diabetes.
6. TUNEL staining in diabetic rat neuroretinal cells was increased and IGF-I administration prevented this increase.
7. Phospho-Akt staining in diabetic rat neuroretinal cells was increased and IGF-I

administration prevented this increase.

8. IGF-I administered subcutaneously acted across the B-CNS-B and prevented impaired learning and memory and reduced protein content in diabetes.

9. An anti-IGF-II antiserum inhibited learning and memory in normal adult rats.

II. CONCLUSIONS AND SIGNIFICANCE:

A. Insulin-like Growth Factor-I Can Prevent Cognitive Impairment in Diabetes: We report here that IGF-I administration normalized overall performance in the Morris water maze, a widely used measure of spatial learning and memory in rats. Dissection of water maze performance leads to the consideration of factors such as sensory competence, task comprehension, motivation, acquisition of information, storage of information, information retrieval, utilization of information, and altered behavior dependent on competence of sensory-motor pathways. IGF-I may have more or less of an effect on all or some of these water maze performance factors which is not surprising in light of brain atrophy. The actions of IGF-I selectivity on learning/memory mechanisms remains a matter for further study. The data here show that IGF-I treatment clearly prevented the impaired behavior of diabetic rats in the water maze.

These findings are of great clinical importance. IGF therapy in diabetic patients may reduce cognitive impairments and possibly reduce the risk of progressing to dementia. Current drugs for dementia act by enhancing existing cholinergic performance. However, all or virtually all of these drugs do not prevent progression of mental deterioration. Both Type 1 and Type 2 diabetic patients show cognitive impairments

(Franceschi et al., 1984; Perlmuter et al., 1984; Helkala et al., 1995; Mooradian, 1997) and cognitive impairments decline over time in adults with Type 1 diabetes (Ryan et al., 1993). Type 2 diabetic patients have a two-fold increased risk of developing dementia (Ott et al., 1999). Based on cognitive impairment and brain atrophy studies, Type 1 diabetic patients are probably at a greater risk of developing dementia, although this study has not yet been done. Generally, diabetic patients have been excluded in population studies of Alzheimer's disease, and the diabetic group is under studied.

B. Brain Atrophy in Diabetes Is in Part Due to Reduced Protein, mRNA, and rRNA Content and IGF-I Administration Prevented Reduced Protein Content: Brain atrophy in diabetes is observed by magnetic resonance imaging (Dejgaard et al., 1991; Araki et al., 1994) and computerized tomography (Soininen et al., 1992). Autopsy of diabetics reveals structural lesions, including axonal loss and the degeneration of ganglion and cortical neurons (Reske-Nielsen et al., 1965). The brain ventricles are enlarged, showing shrinkage of brain matter (Dejgaard et al., 1991; Araki et al., 1994). Scattered loss of anterior horn neurons has also been described (DeJong, 1977; Jakobsen and Sidenius, 1980) The early onset and high incidence of central electrophysiological disturbances suggest that encephalopathy is an accompanying complication of diabetes (Pietravalle et al., 1993; Di Luca et al., 1999).

We report here that wet brain weight was reduced in diabetic rats and that brain atrophy includes a reduction in protein, mRNA, and 18S rRNA per brain. Protein is a large percentage of the organic material that comprises the brain. However, since 70% of the brain is water, only a large reduction in protein would be detected as a significant

difference. Protein content per wet weight of brain was also reduced in diabetes indicating that protein synthesis and/or protein stability was reduced. mRNA and 18S rRNA are essential components of protein synthesis. Reductions in both of these may in part explain the reduction in protein per wet brain weight.

Reduced protein content in the brain may in part explain the cognitive impairments observed in diabetes. Inhibitors of protein synthesis can impair learning and memory in rats using avoidance training (Quevedo et al., 1999; Vianna et al., 2001; Naghdi et al., 2003). This indicates that the consolidation of newly acquired information into stable memories requires the synthesis of new proteins.

Administration of IGF-I partially prevented a reduction in total brain protein content and completely prevented a reduction in protein content per wet weight of brain. IGF-I treatment did not have a significant effect on neither poly(A)+ RNA nor 18S rRNA content in the brain. However, multiple factors contribute to protein synthesis. Small effects of IGF-I treatment on several factors that contribute to protein synthesis such as mRNA content or rRNA content could lead to a detectable overall increase in protein synthesis. In fact, IGFs can elevate alpha and beta tubulin mRNAs and 68 KDa and 170 KDa neurofilament mRNAs in SH-SY5Y neuroblastoma cells during neurite outgrowth (Mill et al., 1985; Fernyhough et al., 1989; Wang et al., 1992) that can lead to increased protein synthesis of major cytoskeletal proteins. It is possible that when studying the whole brain, large differences in small populations of mRNA could be masked.

Although IGF-I did not significantly prevent the reduction in wet brain weight in diabetic rats, cognitive dysfunction was prevented as shown in Chapter 2. This suggests

that IGF-I selectively preserved the learning and memory machinery in diabetic rats. It may be difficult to detect biochemical alterations in the learning and memory machinery when it is pooled with the whole brain. This study looked at biochemical pathology in the whole brain minus the hippocampus and alterations in machinery for other functions would be included. Because both whole brain and the hippocampus showed the same degree of atrophy, it is presumed that the same biochemical pathology shown in whole brain applies to the hippocampus, but confirmation is needed. Larger numbers of animals may allow detection of significant differences of the biochemistry examined in this study.

This is the first time to our knowledge that the biochemistry of brain atrophy is characterized in a rat model of cognitive disorder. Brain atrophy is conventionally detected in humans by MRI or CT scans that describe only a reduction in brain volume. The underlying causes of brain atrophy are not understood and these data help further the understanding of brain atrophy. The biochemical pathology associated with brain atrophy is difficult to study. Autopsy samples have variable histories after death and autolysis complicates the interpretation of results.

C. IGF-I Prevented a Loss of Neuroretinal Cells in Diabetes: Preservation of Neurons May In Part Prevent Diabetic Dementia: Increased TUNEL staining in diabetic rats has been described in the hippocampal region of the brain (Li et al., 2002b) and in the retina (Barber et al., 1998). There are reports of cell loss based on electron microscopy studies in other regions of the rat brain in diabetes (Tay and Wong, 1992). Brain size is reduced by 15% after 11 weeks of diabetes (Table 2-1) and because brain

protein is reduced, I would speculate that part of the loss of weight in the brain is also due to loss of cells throughout the brain. The relative contribution of cell shrinkage, cell loss, and relative involvement of neural and glial cells are an important matter that should be studied further.

These data show that IGF-I administration prevented both the increase in phospho-Akt as well as TUNEL immunostaining in retinal cells in diabetic rats. There is a retinal eye barrier, and possibly IGF-I crosses the barrier as it does the B-CNS-B. Whatever the mechanism, subcutaneous IGF-I administration prevented neuroretinal cell death in diabetic rats. These are the first data to show, to our knowledge, that IGF can prevent cell death in the nervous system in the context of diabetes.

Although IGF-I treatment can prevent cell death in neuroretinal cells., atrophy of the brain occurs nonetheless after 12 weeks of diabetes. The mean wet brain weight was greater in diabetic rats that received subcutaneous IGF-I treatment versus diabetic rats receiving only vehicle (Table 2-1), however the difference was not significant. Both diab + veh and diab + IGF-I groups were significantly reduced compared to the non-diabetic group. Assuming water is 75% of brain weight, and protein is 10% of wet weight, a 30% prevention of brain protein loss would change wet brain weight by only 3%, and this difference would be difficult to detect. These studies should be extended to measure dry as well as wet weights.

These data are of significant clinical importance because IGF treatment may reduce the rate of brain atrophy in diabetes. IGF-I may prevent an overall loss of cells in the brain as its neuroprotective effects are demonstrated in neuroretinal cells. IGF-I may

also reduce hippocampal atrophy by preventing reduced neurogenesis. Hippocampal neurogenesis is increased by IGF-I in hypophysectomized rats that received IGF-I treatment (Aberg et al., 2000) and transgenic mice that over express IGF-I (O'Kusky et al., 2000). Exercise-induced increases in hippocampal neurogenesis are suggested to be mediated by circulating IGF-I (Trejo et al., 2001) albeit elevated serum IGF-I levels were not shown. IGF's may reduce the loss of cells and possibly prevent reduced neurogenesis in the hippocampus of diabetes thereby inhibiting the rate of hippocampal atrophy and possibly delay the onset of hippocampal atrophy. The time course of hippocampal atrophy in diabetic rats receiving IGF-I treatment or vehicle would need to be investigated to answer this question.

D. IGF-I Preserves Brain Function in Diabetes in Spite of Hyperglycemia and an Overall Catabolic State: Neurological complications may arise in diabetes as a consequence of hyperglycemia. This could lead via aldose reductase to elevated polyol levels in the peripheral nervous system, but, disappointingly, peripheral neuropathy has not been prevented in the many clinical trials with aldose reductase inhibitors. One may not exclude the potential contribution of nonenzymatic glycation that might alter the function of proteins. Systemic hyperglycemia is evident within one day of administration of streptozotocin under the conditions of the present study (Wuarin et al., 1996; Zhuang et al., 1996). A persistent hyperglycemia is observed in measurements at 1, 4, 7, 14, and 28 days. Glucose levels remained elevated in the serum of diabetic rats at 12 weeks in the present study, and hyperglycemia was not attenuated by IGF-I treatment (Table 2-1).

IGF-I administration prevented cognitive impairments, partially prevented

reduced protein content per brain and completely prevented reduced protein per wet brain weight in diabetic rats despite unabated hyperglycemia. IGF-I treatment had no effect on severity of hyperglycemia or reduced body weight in diabetic rats (Table 2-1). Body weight is a sensitive index of poor metabolic control. IGF-I or IGF-II administration, despite unabated hyperglycemia in experimental diabetes, can prevent or reverse various neurological disturbances including impaired sciatic nerve regeneration (Ishii and Lupien, 1995; Zhuang et al., 1996), hyperalgesia (Zhuang et al., 1996; Zhuang et al., 1997), neuraxonal dystrophy (Schmidt et al., 1999) and, reduced IGF-II mRNA levels in brain (Armstrong et al., 2000). A loss of IGF activity, rather than hyperglycemia, may confer the primary risk for cognitive disorder in diabetes, albeit long-term hyperglycemia, microangiopathy and cerebrovascular hemorrhage may further aggravate this risk in chronic disease. These observations together are of clinical interest, because they support the hypothesis that IGF administration can prevent peripheral diabetic neuropathy as well as central neurological disturbances even in poorly controlled diabetic patients. They also point to important limitations of the hyperglycemia hypothesis as it applies to the nervous system.

Because IGF administration prevented cell death in rats diabetic for more than 11 weeks, cell death is possibly not due to hyperglycemia nor its secondary consequences per se. Alternatively, the decrease in neurotrophic IGF levels in diabetes may be a more substantial risk factor for neuron loss. IGF-I and IGF-II can prevent neuron loss *in vitro* (Recio-Pinto et al., 1986; Aizenman and de Vellis, 1987) and *in vivo* (Neff et al., 1993; Fernandez et al., 1998; Pu et al., 1999), and anti-IGF antiserum can increase neuron death

in non-diabetic animals (Pu et al., 1999). Anti-IGF antiserum can also inhibit learning/memory as shown in Chapter 2. Cell loss in the nervous system is additionally observed in IGF-I knock-out mice (Beck et al., 1995).

E. Systemically Infused IGF-I Preserves Brain Function in Diabetes: The uptake of circulating IGF-I into CSF is saturable, suggesting that there is an uptake carrier at the B-CNS-B (Armstrong et al., 2000). This uptake shows independence from the IGF type 1 receptor, type 2 receptor, as well as IGF sequestration to IGF binding proteins (Pulford and Ishii, 2001). It is calculated that the normal circulating concentration of IGF-I may contribute substantially to the concentration of IGF-I detected in CSF, and this provides critical support for the hypothesis that circulating IGF supports ongoing functions in the adult mammalian central nervous system.

The subcutaneous administration of IGF-I can prevent poor water maze performance, reduced brain protein content per wet weight of brain, and prevent increased neuroretinal apoptosis in diabetes, showing that IGF-I can preserve brain function across the B-CNS-B. The impermeability of the B-CNS-B is well maintained in diabetes, albeit carrier-mediated transport of some metabolites may be altered (for review see Mooradian, 1997). Because IGF is taken up from blood into CSF, the clinical risk associated with the need to drill an access hole through the skull to deliver a neurotrophic IGF protein (7.5 kDa) may be averted.

F. The Long Term Diabetic Rat Provides a Model for Diabetic Dementia: These data show that after 12 weeks of diabetes, the brain undergoes severe atrophy resulting in a 15% reduction in wet brain weight (Table 2-1). The hippocampus also is

reduced by 15% in wet weight. The reduction in brain weight is not entirely due to loss of water content because the content of protein, mRNA, and 18S rRNA are reduced. Apoptosis that probably occurs throughout the brain but is only shown here in neuroretinal cells would also contribute to brain atrophy. Brain atrophy is not observed after only two weeks of diabetes since there is no difference in wet brain weight between diabetic and non-diabetic rats (Wuarin et al., 1996), and TUNEL staining is not elevated in the neuroretina (data not shown).

The long-term Type 1 diabetic rat can provide a useful model for studying the molecular and cellular effects of brain atrophy. Ultimately, the causation of brain atrophy may be determined. This could possibly lead to therapy that could prevent brain atrophy and/or diabetic dementia. However, brain atrophy can be dissociated from cognitive impairment because IGF-I can overcome learning and memory impairment despite ongoing brain atrophy in diabetic rats.

G. Endogenous IGF Regulates Learning and Memory: The present study shows that infusion of an anti-IGF antiserum into the lateral ventricles inhibits learning/memory in non-diabetic rats in a passive-avoidance test (Fig. 5-2). Infusion of IGF binding protein-6, also inhibits learning/memory in this test (Lupien et al., 2000). Hippocampal lesions prevent passive-avoidance learning (Cogan and Reeves, 1979). Therefore, brain IGF is essential for learning/memory in this hippocampus-based test. This suggests that the support of learning/memory is among the neurobiological roles served by IGFs in the mature mammalian brain. These data are consistent with a model in which brain IGFs help to maintain central nervous system functions, and a loss of IGF

activity may confer increased risk for cognitive decline in diabetes and progression to dementia.

IGF may support learning/memory by enhancing synaptic plasticity, in part, by supporting the formation of neurites and synapses. IGF increases tubulin and neurofilament mRNAs (Mill et al., 1985; Fernyhough et al., 1989; Wang et al., 1992), and the encoded proteins are major axonal cytoskeletal elements. The onset and termination of synaptogenesis is temporally correlated with IGF-II gene expression in perinatal rats (Ishii, 1989). IGF gene expression is increased in nerve Schwann cells (Glazner et al., 1993; Pu et al., 1995) and muscle (Glazner et al., 1994; Pu et al., 1999) following nerve crush injury, and supports nerve regeneration (Kanje et al., 1989; Near et al., 1992; Glazner et al., 1993). IGF increases the sprouting of nerve terminals (Caroni and Grandes, 1990) and numbers of dendritic spines (Nieto-Bona et al., 1997).

Activation of the NMDA receptor is linked to the induction of long-term potentiation (LTP) associated with the formation of memory possibly through increased synaptic strength (Bliss and Collingridge, 1993; Barnes, 1995). An NMDA receptor antagonist can block LTP formation as well as learning/memory (Davis et al., 1992). There is a reduction in immunoreactive NMDA receptors in the postsynaptic densities in the hippocampus of diabetic rats (Di Luca et al., 1999), and this is associated with their impaired induction of LTP (Biessels et al., 1996). The decline in IGF support in diabetes might result in reduced NMDA receptor activity which in turn impairs the formation of LTP. A caveat, however, is that the relationship of LTP to physiological processes involved in learning/memory remains to be clarified.

III. FUTURE EXPERIMENTS:

A. Effect of Diabetes on Neuronal Cell Loss in Diabetic Hippocampus: We have shown here that TUNEL staining, a marker for apoptosis, is increased in the neuroretina of diabetes. Apoptosis also occurs in the hippocampus in diabetes. It is possible that an overall cell loss in addition to cell shrinkage occurs in the diabetic brain including the hippocampus but an experiment to show this is necessary. The hippocampus is essential to at least short-term spatial learning and memory and declarative memory. Loss of cells in the hippocampus may contribute to impaired learning and memory as well as contribute to brain atrophy that could lead to diabetic dementia. It is of interest to test the hypothesis that the total number of cells and the total number of neurons in the hippocampus are reduced and this reduction can be prevented by subcutaneous IGF-I administration.

To test this hypothesis, the experiment would require the use of rats diabetic for 12 weeks that receive either IGF-I (20 μg/day/rat) or vehicle for the final eight weeks and an age-matched non-diabetic group. After 12 weeks of diabetes, transcardially perfuse the rats with 4% paraformaldehyde. Section the hippocampus, 20 μms thick, and stain one out of every 12 sections for neurons with a neuron-specific marker such as doublecortin (Santa Cruz Biotechnology Inc.). The secondary antibody would be conjugated to alkaline peroxidase (Vectastain Elite ABC kit) and reacted with diamino-benzidine (Novocastra) to color the cells brown. Counterstain the sections with cresyl violet that stains Nissl substance. Use the optical fractionator method to count the total number of cells and the total number of neurons in each section. The relative number of

neurons would be compared between groups because with cell loss, reactive gliosis may occur resulting in glial proliferation.

B. Effect of Diabetes and IGF-I Administration on Protein Synthesis in the Brain: We have shown that the total protein content in diabetic rat brain is reduced. This shows that the reduction in the weight of diabetic brain is not entirely due to loss of water content. mRNA and 18S rRNA are essential components of protein synthesis and are also reduced in diabetes. The protein content per wet brain weight is reduced in diabetes suggesting that protein synthesis and/or stability is reduced. This reduction can be prevented by subcutaneous IGF-I administration. It is of interest to test the hypothesis that protein synthesis is reduced in diabetes and that IGF-I treatment can prevent such a reduction.

To test this hypothesis, the experiment would require the use of rats diabetic for 12 weeks or less that receive either IGF-I (20 μg/day/rat) or vehicle for the final eight weeks and an age-matched non-diabetic group. After 12 weeks of diabetes, euthanize the rats and excise the hippocampus. Immediately section the hippocampus coronally and place it in tissue culture media. Add radio-labeled amino acids to the media and incubate for 1 hour at 37°C. Radioactive amino acids will incorporate into newly synthesized proteins. Remove the sections from the media and perform a cold 5% TCA precipitation to remove unincorporated radioactive amino acids. Transfer to a vial and measure the amount of radioactivity per mg of protein in a scintillation counter. Compare the amount of radioactivity between groups to determine if there are any changes in the amount of protein synthesis.

A reduction in protein synthesis in diabetic rat hippocampi could in part explain the observed cognitive impairments in diabetes, since protein synthesis is essential for learning and memory. IGF-I administration prevented cognitive impairments in diabetic rats. This could be explained in part, if IGF-I prevented a reduction in protein synthesis in diabetic rat hippocampi.

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