Hypoglycemic Seizures in Juvenile Rats: Acute Mortality is Associated with Severe Seizures in Diabetic and Non-diabetic Subjects

By

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
   Physiology
   University of Toronto

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Master of Science

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Abstract

Iatrogenic hypoglycemia is a limiting factor for managing diabetes mellitus and can have severe outcomes such as seizures and coma. Although several studies have investigated the central nervous system consequences of hypoglycemia, the effects of seizures, as well as possible treatment strategies, have yet to be elucidated in juvenile animals. The objective of this thesis was to establish an in vivo model of severe hypoglycemia and seizures in juvenile diabetic and non-diabetic rats. In both groups there existed a similar blood glucose threshold for seizures, and mortality only occurred following severe seizures, particularly with repeated seizures that were unresponsive to treatment. While the administration of anticonvulsants temporarily mitigated seizures, glucose administration was required to prevent mortality. Abnormalities in the hippocampal and brainstem electroencephalograms (EEG) were observed in hypoglycemic animals without a clear correlate to convulsive activity.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>anticonvulsant treatment</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
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<tr>
<td>BB</td>
<td>bio breeding</td>
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<tr>
<td>BG</td>
<td>blood glucose</td>
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<td>CA1</td>
<td>hippocampus</td>
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<td>CB</td>
<td>controls</td>
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<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
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<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
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<tr>
<td>DKA</td>
<td>Diabetic Ketoacidosis</td>
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<td>DN</td>
<td>Diabetic Neuropathy</td>
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<tr>
<td>ECoG</td>
<td>Electrocorticogram</td>
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<tr>
<td>ECS</td>
<td>extracellular space</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>FDG-PET</td>
<td>Fludeoxyglucose – positron emission tomography</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GADA</td>
<td>glutamic acid decarboxylase autoantibodies</td>
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<tr>
<td>GLUT</td>
<td>Glucose Transporter</td>
</tr>
<tr>
<td>IA-2A</td>
<td>insulinoma associated 2 autoantibodies,</td>
</tr>
<tr>
<td>IAA</td>
<td>insulin autoantibodies</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell autoantibodies</td>
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<tr>
<td>IDDM</td>
<td>Insulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>ISPAD</td>
<td>International Society for Pediatric and Adolescent Diabetes</td>
</tr>
<tr>
<td>LETL</td>
<td>Long Evans Tokushima lean</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NS</td>
<td>non-seizing</td>
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<tr>
<td>MCT</td>
<td>monocarboxylic acid transporter</td>
</tr>
<tr>
<td>MRF</td>
<td>mesencephalic reticular formation</td>
</tr>
<tr>
<td>PASL</td>
<td>Pulsed Arterial Spin Labeling</td>
</tr>
<tr>
<td>PN</td>
<td>Postnatal</td>
</tr>
<tr>
<td>PPTg</td>
<td>Pedunculopontine tegmental nucleus</td>
</tr>
<tr>
<td>SGLT</td>
<td>Sodium-coupled Glucose Transporters</td>
</tr>
<tr>
<td>SLEs</td>
<td>seizure-like events</td>
</tr>
<tr>
<td>S+M</td>
<td>seizure + mortality</td>
</tr>
<tr>
<td>SNR</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>S+S</td>
<td>seizure + survival</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin/ diabetic</td>
</tr>
<tr>
<td>SWDs</td>
<td>spike wave discharges</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxidative species</td>
</tr>
<tr>
<td>ZnT8A</td>
<td>recently described zinc transporter autoantibodies</td>
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1.1 Energy Metabolism in the Brain

1.1.1 Glucose Metabolism

The brain has a high resting metabolic rate, third only to the heart and kidney cortex (McKenna et al, 2006) and this rate increases during localized brain activation. Being unable to metabolize fatty acids, the brain is heavily dependent on glucose, as well as ketones, as sources of energy.

Unlike muscles and adipocytes where insulin is directly responsible for glucose uptake through the activation of GLUT4 (Glucose Transporter) receptors, GLUT1 receptors in the brain are unresponsive to insulin. While GLUT4 receptors are also present in the brain, brain glucose uptake appears to occur independently of insulin, relying on GLUT1 receptors, present on capillaries that act to allow circulating glucose in the blood to cross the blood brain barrier. This action is crucial since the brain stores little glucose in the form of astrocytic glycogen (Brown, 2003). In fact, brain tissue consumes about 600 µM glucose/g/h whereas the concentration of brain glycogen is approximately 0.5–1.5 µM/g of tissue. These data suggest that in the absence of glucose, glycogen would be consumed in a few minutes (Brown and Ransom 2007).

Glucose is then transported into cells either by SGLT (Sodium-coupled Glucose Transporters) or GLUT3 receptors (McCrimmon and Sherwin, 2010). As a result of this transport chain, brain glucose levels can be as low as 10-30% of that in blood depending on the region (Gonzalez et al, 2009). SGLT receptors possibly work as non-metabolic sensors and can uptake glucose in conditions of high glucose levels such as diabetes and obesity (Gonzalez et al, 2009). Other glucose receptors present in the brain are GLUT2 receptors in astrocytes necessary for transport of catabolized glycogen (Marty et al, 2005) and GLUT 5 transporters in microglia (McCrimmon and Sherwin 2010).
1.1.2 Sources of Energy Substrate

As previously mentioned, the brain stores minimal glucose in the form of astrocytic glycogen (6-12µmol), partly due to limited intracranial space (Dringen et al, 1993; Brown and Ransom, 2007; Gruetter, 2003; McCall, 2004; Seaquest et al, 2001). A study using 13C nuclear magnetic imaging measured an increase in brain glycogen metabolism of 15% during hypoglycemia (Gruetter, 2003) and this elevation has neuroprotective effects that were prevented by blocking glycogen metabolism using the glycogen phosphorylase inhibitor, isofagomine (Brown et al, 2005). In addition, manipulations that either increased or decreased glycogen content influenced the time that the stimulus-evoked compound action potential (reflecting axonal function) could be maintained in a rat optic nerve when deprived of glucose (Brown and Ransom, 2007).

Astrocytic glycogen is broken down into lactate because astrocytes, unlike peripheral cells such as hepatocytes, possess lactate dehydrogenase instead of glucose-6-phosphatase. Lactate uptake is then facilitated via monocarboxylate transporters (MCT) present on neuronal membranes. Neurons rely on this energy pathway when brain energy is low (Brown and Ransom, 2007) and the administration of MCTs can prevent the development of hypoglycemia-induced cognitive dysfunction (McCrimmon, 2012). The consumption of lactate till its depletion is more efficient than stimulating glucose-sensing neurons in the hypothalamus to induce energetically demanding activities such as liver glycogen catabolism or foraging for food (Gonzalez et al, 2009).

1.1.3 Biochemical Changes in Hypoglycemia

In humans, glucose concentration in brain interstitial fluid is only a fraction of plasma glucose (Abi-Saab et al, 2002). This implies that as the plasma blood glucose is in the normal range of 3.5-7.1mM, the corresponding normal brain concentrations range from approximately 0.8–2.3 mM (Gruetter et al, 1998) and during hypoglycemia these levels approach zero (Suh et al, 2007).

Recordings with ion-sensitive electrodes show that during aglycemia, there is an alkalization of the extracellular space (ECS) (Brown et al, 2001). This is in contrast to anoxia or ischemia where acidosis results (Ransom and Philbin, 1992). Under normoglycemic conditions
there is a constant efflux of lactate from astrocytes (lactate is co-transported with \( \text{H}^+ \)), but this alkalization suggests that during aglycemia the lactate source is depleted and the ECS concentration of lactate and \( \text{H}^+ \) is decreased (Brown and Ransom, 2007).

Critically low glucose can upset the balance between inhibition and excitation of neuronal networks. The loss of high-energy substrates leads to the release of excitatory amino acids that promote hyperexcitability and consequent excitotoxicity (McCall, 2004). In addition, \( K_{\text{ATP}} \) channels also play a neuroprotective role. These channels open and hyperpolarize neurons during energy depletion to stop expenditure on action potential firing (Ghasemi et al, 2010). Expression of these channels decrease after an overnight fast in rodents, therefore contributing to neuronal excitability and possibly to hypoglycemic seizures (Velsek et al, 2008).

1.2 Seizures

1.2.1 Definition

Before I further discuss the effects of diabetes and hypoglycemia on seizures, I will make the distinction between seizures and epilepsy.

The International League Against Epilepsy defines:

a) Epileptic seizure as “a transient occurrence of signs and/or symptoms due to abnormal, excessive or synchronous neuronal activity in the brain.” (Fisher et al, 2005):

b) Epilepsy as “a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures” and by “the neurobiologic, cognitive, psychological, and social consequences of this condition” (Fisher et al, 2005)

The definition of epilepsy requires the occurrence of at least two epileptic seizures.

While it is well established that seizures are a disorder where altered neural substrates result in synchrony (Zhong et al, 2011), this phenomenon can have several causes. These were previously classified as idiopathic, symptomatic or cryptogenic. However recent alterations have been proposed to more accurately represent the causes of seizures. It has been suggested that the
categories be changed to structural or metabolic, genetic and unknown (Berg et al, 2010). Hypoglycemic seizures, previously characterized as symptomatic, should now be classified as metabolic.

1.2.2 Seizures in the Immature Brain

In the developing brain, excitation is predominant and there is an overproduction of synapse and spine density. Ion channels are expressed at levels that promote excitation whereas inhibition develops later (Rakhade and Jensen, 2009). The propensity for seizures or seizure-like activity in the immature brain has been shown in several experimental models, including kainic acid, electrical stimulation, hypoxia, penicillin, picrotoxin, GABA receptor antagonists, and increased extracellular potassium (Ben-Ari and Holmes, 2006).

In adult male rats, the substantia nigra pars reticulata (SNR) has two topographically different regions that have opposing effects in regulation of excitability. The GABA_\text{A}-sensitive “anticonvulsant” region is located in the SNRanterior, whereas the GABA_\text{A}-sensitive “proconvulsant” region is in the SNRprior (Veliskova et al, 2004). Age-related differences in seizure susceptibility may be due to functional differences in the SNR (Moshe et al, 1995). Muscimol and THIP (GABA agonists) are unable to suppress seizures in 2-week old rats (Moshe et al, 1995). A potential reason is that in rats, aged 15-21days, only that proconvulsant region is present. The switch in SNR effects on seizures may occur in rats around 30-35 days (Moshe et al, 1995). In 30 day olds there is more control of seizures when muscimol is infused in the anterior-medial SNR. The segregation between the anticonvulsant anterior-medial region and the proconvulsant posterior-lateral region, of the SNR, likely occurs during this stage of development (Moshe et al, 1995, Sperber et al, 1999). It should be noted that female rats at any age did not exhibit proconvulsant effects with muscimol infusions to the SNR and this developmental difference appears to be mediated by testosterone. The proconvulsant SNR is not expressed when testosterone is absent in the immediate postnatal period (Veliskova et al, 2004).

Seizures occurring in neonatal rats can have prolonged changes in neuronal networks altering the inhibition/excitation balance and, reducing seizure threshold that persists into adulthood (Iseava et al, 2010). Frequent and prolonged seizures in the developing brain may intervene with developmental programs and lead to inadequate construction of cortical networks rather than induction of neuronal cell loss. This difference is because developing networks, up to
about two weeks in rodents, are resistant to damage after prolonged seizures. The lack of cell loss is likely due to reduced vulnerability to glutamate toxicity (Ben-Ari and Holmes, 2006). Though, recurrent seizures can impair neurogenesis of the immature brain, it is unknown whether there are long-term detrimental effects (Ben-Ari and Holmes, 2006). In part, this could be due to the down-regulation of GABA transmission observed in rats several days after the induction of neonatal seizures (Iseava et al, 2009). A single seizure in p10 rats resulted in Purkinje cell damage and an up-regulation of AMPA receptor subunits (Lomoio et al, 2010). Moreover, GABA has an excitatory effect up to about 14 postnatal days in rats as well as the third trimester in primates (Ben-Ari and Holmes, 2006).

The decrease in AMPA receptor subunit, GluR2, in the dentate gyrus after pilocarpine-induced seizures (Zhang et al, 2004), and in the hippocampus and neocortex after hypoxia-induced seizures, lowered the threshold for seizures. This is possibly due to increased calcium influx and channel conductance (Sanchez et al, 2001). In addition, the increased expression in the immature brain of NR2B, NR2D and NR3A subunits all contribute to increased NMDAR-mediated calcium influx and lower the threshold for seizures and excitotoxic hypoxic–ischemic injury (Rakhade and Jensen 2009).

1.2.3 Electroencephalogram

The electroencephalogram (EEG) is a measure of the extracellular potential, which is the superimposition of neuronal currents generated in a given area. It can be recorded from the scalp (EEG), subdurally on the cortical surface (electrocorticogram; ECoG), and through small electrodes in the brain (intracranial EEG or Local Field Potential; LFP) (Buzsaki et al, 2012).

The first EEG published was by Hans Berger in 1929. It presented the first recorded brain waves from an intact human scalp. The present day convention defines brain waves in the following frequency ranges: delta: 0.1-3.5Hz; theta: 4-7.5 Hz; alpha: 8-13 Hz; beta: 14-30 Hz; gamma: more than 30 Hz. (Somjen, 2004).

Intracranial EEGs or LFPs can be used to detect activity that may not be picked up by scalp EEG recordings (Buzsaki et al, 2012). In humans, intracranial EEG recordings have recorded focal high-frequency discharges in regions of seizure onset and propagation (Norden and Blumenfeld, 2002). In animal models, EEGs can be used to detect electrographic seizure
activity, such as spike wave discharges (SWDs) in absence seizures and in metabolic models such as hypoxia, as well as determine seizure focus (Del Campo et al, 2009b; Wais et al, 2009).

1.3 Clinical Studies

1.3.1 Pathophysiology of Diabetes

Diabetes affects over 350 million people worldwide (Ghasemi et al, 2010) and Type 1 Diabetes or Insulin Dependent Diabetes Mellitus (IDDM) accounts for 5-10% of diabetic patients (Daneman, 2006). As of 2009, there were approximately 480,000 children and adolescents under the age of 15 affected by diabetes worldwide and this number is expected to increase by 3% annually (IDF: http://www.idf.org/diabetesatlas/diabetes-young-global-perspective). Since its discovery in 1922, insulin has been the only treatment to manage IDDM.

IDDM is an autoimmune disease where activated CD8+ T-cells are directly responsible for causing β-cell death. This results in the release of additional intracellular antigens and stimulates additional autoreactive T-cells that amplify the initial autoimmune response. Autoantibodies associated with IDDM include (1) islet cell autoantibodies (ICA), (2) glutamic acid decarboxylase autoantibodies (GADA), (3) insulinoma associated 2 autoantibodies (IA-2A), (4) insulin autoantibodies (IAA), and (5) recently described zinc transporter autoantibodies (ZnT8A). β-cells destruction results in hypoinsulinemia thus impairing the uptake of glucose by muscles and adipocytes (Brown and Ransom, 2007). Symptoms of IDDM in addition to hyperglycemia include weight loss, polyuria, and polydipsia (Gan et al, 2012).

Diabetic Ketoacidosis (DKA) is a complication caused by the deficiency of insulin. The inability to uptake glucose results in lipolysis causing the formation of ketone bodies (B-hydroxybutyrate and acetoacetate), which provide an alternate source of energy but increase metabolic acidosis (Gan et al, 2012). MCTs along the blood brain barrier may be upregulated in IDDM to allow the uptake of acetate, lactate, and ketone bodies (Brown and Ransom, 2007). Hyperglycemic Hyperosmolar Syndrome, characterized by extreme blood glucose elevation and hyperosmolality (>330 mOsm/kg), has a high mortality rate (Gan et al, 2012).

The more long-term complications of diabetes include, Diabetic Neuropathy (DN), a set of heterogeneous clinical syndromes that affect distinct regions of the peripheral nervous system
(Vinik, 2006). 10% of children with IDDM have symptoms of peripheral DN but the frequency of subclinical neuropathy is unknown. DN is a result of axonal loss, previously postulated to affect the longer fibers first. It has been recently found that small fiber dysfunction is also a factor presenting initially as pain, hyperalgesia and allodynia in lower limbs and later as reduced sensation (Vinik, 2006). These detrimental conditions associated with IDDM make it crucial to maintain euglycemic levels with the appropriate insulin dosage throughout the lifetime of the patient.

1.3.2 Counterregulation

Insulin delivered exogenously is not subject to the normal physiological feedback regulation, and can therefore result in hypoglycemia despite intact counterregulatory mechanisms. Increased glycogen content after hypoglycemia may also contribute to suppression of counterregulation during subsequent hypoglycemia (McCrimmon 2012). As well, recurrent moderate hypoglycemia diminishes the ability to recognize the warning symptoms (hypoglycemia unawareness) and decreases the counterregulatory response to subsequent hypoglycemic events (hypoglycemia-associated autonomic failure), thus jeopardizing patient safety (Cryer, 2004; Inzucchi & Sherwin, 2007; Puente et al, 2010).

Counterregulation in non-diabetic individuals is induced to increase glucose delivery to the brain. A homeostatic response that suppresses endogenous insulin, increases secretion of counterregulatory hormones like glucagon and epinephrine and decreases glucose utilization in the periphery (Cryer, 2004).

In the context of diabetes, decreasing levels of glucose are unable to regulate exogenous insulin. Consequently, glucagon concentrations do not increase to stimulate hepatic glucose production. The adrenaline response is unable to compensate and is eventually reduced due to sensitization by recurrent hypoglycemic incidents. It is postulated that the attenuated adrenaline response causes the clinical syndrome of hypoglycemia unawareness. The diminished warning symptoms of hypoglycemia compromise the patient’s ability to correct hypoglycemia by ingesting glucose (Cryer 2002).
1.3.3 Hypoglycemia in Diabetes

Iatrogenic hypoglycemia is a major limiting factor in the management of IDDM (Cryer, 2004, Blassetti et al, 2011, Katz, 2012). Achieving tight glycemic control is a balancing act between insulin and counter-regulatory hormones such as such as glucagon, epinephrine and norepinephrine (Cryer 2004). According to the Diabetes Control and Complications Trial (DCCT), both conventional and intensive insulin therapy resulted in hypoglycemia in patients but with intensive insulin therapy the risk of hypoglycemia was increased three-fold. Within conventional and intensive treatment groups (has been demonstrated to achieve the goals of reducing mean blood glucose and the risk of the development and progression of the microvascular and neurologic long-term complications of IDDM), the number of prior episodes of hypoglycemia was the strongest predictor of the risk of future episodes (DCCT). Patients who experienced severe hypoglycemia were at increased risk of subsequent episodes with approximately 30% of individuals incurring a second hypoglycemic episode within 4 months (DCCT).

The average individual with type 1 diabetes experiences about two hypoglycemic episodes per week and this figure has been consistent over the last 20 years (McCrimmon and Sherwin, 2010). Severe hypoglycemia has an annual incidence of 1.0-1.7 episodes per year. While non-diabetic individuals are able to maintain glucose homeostasis through counterregulation, hypoglycemia is a common occurrence in Type 1 diabetics. It almost exclusively occurs when excess insulin is administered to control hyperglycemia. The resultant hyperinsulinemia blocks the peripheral generation of fuels such as hepatic production of glucose through glycogenolysis (McCrimmon, 2012).

Recent studies have shown that hypoglycemia mediated by the loss of signaling between α and β cells of the pancreas is regulated at the level of the ventromedial hypothalamus (McCrimmon and Sherwin, 2010). As mentioned, hypoglycemia unawareness is associated with a diminished adrenaline response. FDG-PET showed a significant decrease in adrenaline response (0.77 ± 0.39nM vs. 7.52 ± 2.9nM) in patients exhibiting asymptomatic hypoglycemia (Cranston et al 2001). Patients do not treat themselves, as they are unable to recognize early hypoglycemia, leaving it uncorrected. This failure in homeostatic mechanisms can then lead to more severe outcomes like seizures. (Cranston et al 2001).
1.3.4 Hypoglycemic Seizures

As a result, mismanaged insulin treatment can lead to hypoglycemia-induced seizures and coma, with generalized seizures being the major acute complication (Lapenta et al, 2010) occurring particularly in children (Davis et al, 1997) and adolescents (Svoren et al, 2003). Furthermore, devastating effects, such as the “dead in bed” syndrome, in part due to hypoglycemia (Secrest et al, 2010), occur approximately 3 times more frequently in young people with diabetes than in those without (Heller, 2002).

More recently, clinical studies have been able to show electrographically distinct features for hypoglycemic seizures. In a diabetic woman with an epileptic seizure, monomorphic activity of 5-6Hz slow-wave frequencies in the right temporal lobe was reported. However there was an absence of interictal activity as well as other ictal symptoms, making them distinct from non-hypoglycemic complex-partial seizures (Lapenta et al, 2010). A clinical trial investigating the EEG of patients with a history of severe hypoglycemia showed a reduction of spectral power in β (Howarka et al, 2000).

Lastly, a clinical study assessed seven nocturnal seizures in three diabetic children during hypoglycemia. All seven were tonic-clonic seizures of 3-20 minute duration that involved automatisms of the face and limbs. However, no definite seizure focus was determined and only one of the seven seizures was associated with EEG slowing in the left hemisphere (Lahat et al, 1995). Hypoglycemic seizures are often not recognized as seizures as they manifest similarly to other disorders of hypoglycemia such as autonomic symptoms and impaired consciousness (Lapenta et al, 2010).

1.3.5 Effects of Hypoglycemia on the CNS

Hypoglycemic neuronal damage has been observed to proceed from rostral to caudal brain regions, which may correlate with the concentration and distribution of brain glycogen (Brown and Ransom, 2007). This damage is observed in hypoglycemia concomitantly with EEG isoelectricity, which requires levels of blood glucose < 1.0mM (Auer, 1989). The cortex and CA1 and DG of the hippocampus appear to be more susceptible to this damage while the brainstem and cerebellar regions remain resistant. A hypoglycemic episode can result in the suppression of the counterregulatory hormone response to a second episode 12 – 24 hours later.
(McCrimmon, 2012). Yet hypoglycemia may trigger a hypoglycemic tolerance or habituation to limit cellular damage. In 2010, Puente et al demonstrated this in a study, where recurrent moderate hypoglycemic episodes were protective against neuronal damage caused by a subsequent severe hypoglycemic episode (Puente et al, 2010).

Cerebral blood flow (CBF) increases during hypoglycemia possibly in an attempt to deliver adequate blood glucose to the brain. Diabetic patients showed increased cerebral blood flow at higher blood glucose levels during hypoglycemia than controls. This increase was also found to be greater in children than adults. In children, CBF measurements showed an increase during hypoglycemia especially in the right hemisphere and gray matter. These data may suggest laterality of seizure origin. The left hemisphere may be more susceptible to deleterious effects as there is a reduced ability to increase cerebral blood flow (Jarjour et al, 1995, Tupola et al, 2004). However, other studies do not show this asymmetry (Kerr et al, 1993).

Through imaging studies, mild hypoglycemia has been demonstrated to increase hypothalamic blood flow. In 2008, a study by Musen et al reported activation of the hypothalamus, brainstem, anterior cingulate cortex, uncus and putamen in both diabetic patients and age-matched controls which the authors posit to be regions recruited to respond to hypoglycemia (Musen et al, 2008). Pulsed Arterial Spin Labeling (PASL) confirmed an increase in hypothalamic blood flow during moderate hypoglycemia in nondiabetic individuals as blood glucose begins to decrease and before counterregulation occurs (Page et al, 2009).

Lastly, EEG abnormalities are more frequent in patients with IDDM than the population at large and an abnormal EEG at diagnosis appears to be predictive of future hypoglycemic coma or convulsions (Tupola et al 2004). With the emergence of technology for continuous subcutaneous blood glucose monitoring and the delays associated with readings, it is critical to know how long hypoglycemia can be tolerated before seizures occur (Buckingham et al, 2008).

1.3.6 Hypoglycemia in Children

According to the DCCT, annual rates of severe hypoglycemia in children with IDDM are 5-10%. The International Society for Pediatric and Adolescent Diabetes (ISPAD) Clinical Practice Consensus Guidelines provides no consistent definition for hypoglycemia in children. Generally a blood glucose level between 3.3 and 3.9 mM is considered a risk factor for severe
hypoglycemia. Behaviourally, this is defined as the loss of consciousness, leading to seizures or coma. As previously mentioned, there is increased seizure susceptibility in the developing brain. Therefore it is not surprising that in children, 90% of hypoglycemic episodes result in seizures followed by coma (Davis et al, 1997) with 75% of seizures occurring at night (Buckingham et al, 2008). Diabetic patients appear to have 10 times greater risk of sudden unexplained deaths or the dead-in-bed syndrome (Secrest et al, 2010), potentially the result of seizures (Tattersall and Gill, 1991). In addition, in young children there is a concern of severe permanent neurological deficits following significant hypoglycemia (Buckingham et al, 2008).

1.3.7 Hypoglycemia and Effects on Cognition

Neurological impairments can occur even during moderate hypoglycemia. In patients, auditory and visual reaction time is prolonged when plasma glucose falls below 3.3 mM, and cognitive function begins to deteriorate as plasma glucose concentration falls below approximately 2.5–3.5 mM (Suh et al, 2007).

Young children may be more susceptible to neurological deficits caused by hypoglycemia (Buckingham et al, 2008). At moderate levels, hypoglycemia can impair cognition in both healthy and diabetic individuals (Suh et al, 2007). Seizures have been found to further exacerbate this dysfunction (Kaufmann et al, 1999). Episodes of severe hypoglycemia are associated with deficits in cognition and attention (Hannonen et al, 2003). In particular, patients underperformed in short term memory, verbal memory and had learning difficulties in reading, spelling and math (Hannonen et al, 2003; Kaufmann et al, 1999).

Meanwhile, other studies demonstrate contradictory results (Frier, 2011). In 2000, Howorka et al observed no difference in concentration or attention in IDDM patients with or without a history of severe hypoglycemia (Howorka et al, 2000). Another study showed a reduction in spatial intelligence scores only with repeated hypoglycemic episodes and when the first episode occurred before the age of 5 (Perantie et al, 2008).

1.3.8 Prevention of Hypoglycemia

As previously described, the disruption of counterregulation causes hypoglycemia unawareness resulting in the patients’ inability to intervene and correct the condition. Therefore, methods of detecting moderate levels of hypoglycemia would aid in preventing the
more detrimental effects of severe hypoglycemia. In 2009, Buckingham et al used a closed looped system on subjects with IDDM. A predictive algorithm was utilized to assess the risk of hypoglycemia and the insulin pump would be suspended for 90 min when it was predicted that hypoglycemia would fall below 80 mg/dl. This system was successful in preventing 60-80% of hypoglycemic seizures (McCrimmon, 2012)

1.4 In Vitro Studies

In vitro studies have provided further evidence for the importance of maintaining tight glycemic control in the brain. Both hyper and hypoglycemia have been found to upset the inhibitory/excitatory balance of neural networks and reduce seizure threshold.

A high-concentration glucose medium of 20mM, twice the normal levels, in magnesium-free ACSF enhanced seizure discharges in the rodent hippocampus (Schwechter et al, 2003). Increased spontaneous activity was recorded in CA3 regions of the hippocampus obtained from rats that were hyperglycemic for over four weeks. However, in rats that were diabetic for three weeks or less, this excitability was not observed (Margineanu et al, 1998).

Previous studies have demonstrated attenuation in basal synaptic activity by hypoglycemia (Fan et al, 1988; Kirchner et al, 2006). In contrast to seizures observed in patients during hypoglycemia, low concentrations of glucose (1 or 2mM) in ACSF did induce epileptiform activity in hippocampal slices (Kirchner et al, 2006). More recently, in 2007, Abdelmalik et al demonstrated epileptiform discharges with the perfusion of low glucose to the hippocampus but not the neocortex of young mice (Abdelmalik et al, 2007).

1.5 In Vivo Studies

1.5.1 Models of Diabetes

“Marjorie” the dog, one of the most famous experimental animals in history, was used by Banting and Best in the 1920s as a diabetic model to test insulin (Rees and Alcolado, 2005); the sole treatment for IDDM to date. Since then, animals have been utilized to elucidate the mechanisms and pathophysiology of diabetes in order to achieve a cure. Hyperglycemia, being the most detrimental consequence of diabetes must be modeled to study and manage related
outcomes such as diabetic neuropathy and retinopathy. A partial or complete pancreatectomy is
the most direct method to induce hyperglycemia. Cytotoxic glucose analogues such as
streptozotocin (STZ) and alloxan cause damage to pancreatic β cells (Lenzen and Patten, 1988;
Rees and Alcolado, 2005; Lenzen, 2008). While alloxan is unreliable for induction of diabetes in
vivo, STZ is widely used as a method for inducing diabetes in animal models (Like and Rossini,
1976). Given the appropriate dosage of STZ, fasting blood glucose remains elevated (Like and
Rossini, 1976). However, it should be noted that the above methods of diabetes induction do not
mimic the inflammatory autoimmune process that is the cause of IDDM in most humans (Reddy
et al, 1995).

Therefore, the non-obese diabetic (NOD) mouse and the bio-breeding (BB) rat are
commonly used models. These animals spontaneously develop diseases that present similarities
to IDDM (Rees and Alcolado, 2005). Others such animal models include the LETL (Long
Evans Tokushima lean) rat (Kawano et al, 1991), the New Zealand white rabbit, the Keeshond
dog, the Chinese hamster and the Celebes black ape (Macaca nigra) (Rees and Alcolado, 2005).
In NOD mice, insulitis occurs at 4–5 weeks of age, followed by subclinical β cell destruction
causing decreased insulinemia, and finally diabetes typically presents at around 12 to 30
weeks of age. Unlike human Type 1 diabetes, ketoacidosis is relatively mild and affected animals can
survive for weeks without the administration of insulin (Atkinson and Leiter, 1999). In the BB
rat, weight loss, polyuria, polydipsia, hyperglycaemia and insulinopenia develop at around
12 weeks of age. Ketoacidosis is fatal in these rats and exogenous insulin is required. These
spontaneous models differ from toxin-induced diabetes as immune cells in these animals are
recruited to the insulitis and attack the pancreatic islets (Rees and Alcolado, 2005). As such,
these models better represent the autoimmune dysfunction that results in IDDM in humans.

1.5.2 Brain Damage

Neuronal damage is observed in hypoglycemia concomitantly with EEG isoelectricity.
This requires a blood glucose concentration less than 1.0mM (Auer et al; 1984; Lewis, 1974;
Bree et al, 2009). Previous animal studies of severe hypoglycemia have shown that neuronal
damage initially occurs in the cerebral cortex as well as in the CA1 and dentate gyrus regions of
the hippocampus (Auer et al 1989, Bree et al 2009) followed by neuronal damage in the basal
ganglia and the thalamus. Neurons in the brain stem, the cerebellum, and the spinal cord are
generally spared, as are glial cells and white matter tracts (Auer et al, 1989). Mild, recurrent hypoglycemia can cause synaptic dysfunction in the hippocampus, even in the absence of neuronal death (Yamada et al, 2004).

Diabetes has been demonstrated to exacerbate hypoglycemia-induced damage with STZ rats showing an increased number of fluorojade positive cells, a marker of neurodegeneration, compared to non-diabetic rats and seizures further aggravated this damage (Bree et al, 2009). However, recurrent moderate hypoglycemia appeared to be protective against neuronal damage caused by a subsequent severe hypoglycemic event (Puente et al, 2010).

1.5.3 Glucose Reperfusion Injury

Hypoglycemia-induced neuronal damage is not simply the result of an energy deficiency but other events that are initiated by hypoglycemia. These events include activation of neuronal glutamate receptors, production of ROS, neuronal zinc release, activation of poly(ADP-ribose) polymerase–1, and increased mitochondrial permeability (Suh et al, 2007b). Generation of superoxide species by neuronal NADPH oxidase was greatly increased during glucose reperfusion compared to hypoglycemia (Suh et al, 2007b). NADPH oxidase can be activated by zinc, which is stored in vesicles and released along with glutamate in response to depolarizing stimuli (Somjen, 2004). Zinc can modulate NMDA receptor activity, enhance AMPA receptor-mediated current, or attenuate glutamate transmission and reduce seizure activity. However, elevated extracellular zinc can exacerbate neuronal injury due to excitotoxicity or hypoxia (Somjen, 2004). In addition, brain regions such as the dentate gyrus that are vulnerable to hypoglycemia-induced damage coincidentally contain high concentrations of presynaptic vesicular zinc (Somjen, 2004; Suh et al, 2007b).

1.5.4 Seizures

In addition to excess insulin, metabolic stress such as fasting is associated with downregulation of $K_{ATP}$ channels. The resulting impairment in hyperpolarization may be a predisposing factor for seizure development as fasted animals exhibited an increased propensity for seizures (Velsek et al, 2008). While EEG and imaging studies have demonstrated that structures such as the SNR, pedunculopontine tegmental nucleus and superior colliculus are
involved in the control of hypoglycemic seizures, the origin of these seizures remains unknown (Velsek et al 2008).

Since the SNR has demonstrated control over the motor component of hypoglycemic seizures, a discussion on this region’s functionality in controlling convulsions is useful. GABA sensitive neurons in the SNR regulate seizure susceptibility in rats and these effects are age-dependent (Moshe 1995). Microinfusions of GABA$_A$ agonists have been reported to suppress seizures in some but not all models of epilepsy i.e. pentylenetrazol seizures in adult rats. Furthermore, the SNR mediates seizures in deep brain layers such as the superior colliculus and the pontine reticular formation (Moshe et al, 1995).

1.5.5 EEG and Hypoglycemia

Recent findings indicate that the benefits of glycogen stores, demonstrated in *in vitro* studies, extend to whole animal models as well. Studies have shown that increased intracellular glycogen levels delay the onset of isoelectric EEG, a recognizable sign of trans-membrane ion gradient breakdown. Manipulation of glycogen with the glycogen phosphorylase inhibitor, CP316819, resulted in an increase of brain glycogen, which diminished the pathological effects of insulin-induced systemic hypoglycemia. This phosphorylase inhibitor has the special feature of allowing glycogen breakdown to occur in the face of profound hypoglycemia. The benefit of glycogen was surprisingly strong and preserved brain function for up to 90 min (Suh et al, 2007).

Non-diabetic animal models have shown that observed seizure-like behaviours was poorly associated with electrographic abnormalities recorded in the cerebral cortex and hippocampus. Seizure-like behaviour was observed during the periods of high amplitude slow wave activity, burst-suppression or spiking. Only 1 of 8 animals showed a correlation of behaviour with bursts of epileptiform activity (Del Campo et al, 2009). Similarly, Velsek et al observed that clonic seizures and recurrent barrel rotations were associated with EEG discharges. However, EEG seizures developed later than motor seizures and were recorded almost simultaneously in the cortex, hippocampus, SNR and pedunculopontine tegmental nucleus (PPTg) (Velsek et al, 2008). These data suggest that the sites of seizure origin have yet to be uncovered.
1.6 Rationale

1.6.1 Streptozotocin (STZ) Model of Diabetes

Animal models appear to have similar responses to acute hypoglycemia and develop similar defects as human patients. While the BB rat better represents the inflammatory attack of β cells that occurs in IDDM, the disease presentation is only at around 12 weeks. As such, this model of diabetes cannot be used in younger rats. Surgical resection of the pancreas where glucagon-producing α cells are removed along with β cells could produce confounding effects. The STZ model, where the drug specifically targets β cells and enters them via the glucose transporter (GLUT2), is comparatively more robust than the aforementioned models (Szkudelski, 2001). Though the action of alloxan is also specific to β cells, the dose range for diabetes induction is narrow and slight overdosing can be fatal (Szkudelski, 2001).

STZ is a nitrosurea derivative isolated from *Streptomyces achromogenes* with broad-spectrum antibiotic and anti-neoplastic activity (Rees and Alcolado, 2005) and is used as a chemotherapeutic agent for insulinomas (Lenzen, 2008). It functions as an alkylating agent, transferring a methyl group to guanine, which causes a cascade of events resulting in DNA fragmentation and β cell necrosis (Lenzen, 2008). In addition, STZ liberates nitric oxide that may exacerbate DNA damage (Szkudelski, 2001).

A single STZ dose can cause β cell necrosis within 4 hours and stable hyperglycemia can be observed within 1-2 days of administration (Like and Rossini, 1976). Intravenous or intraperitoneal (IP) administration of 40-60mg/kg is used to induce IDDM in adult rats (Szkudelski, 2000).

1.6.2 Juvenile Model

The increased seizure susceptibility of the immature brain is potentially due to the delay in development of networks that aid in suppressing seizures in adults (Moshe et al, 1995). This increased vulnerability to seizures is evidenced by fever-induced febrile seizures that occur frequently in infants and children but not in adults (Ben-Ari and Holmes, 2006). Similarly, hypoglycemia results in seizures more often in children (Davis et al, 1997) and can cause neurological deficits particularly when they occur in young children (Kaufmann et al, 1999;
Perentie et al, 2008). Despite this, to my knowledge, no model exists of diabetes in young animals that has been used to study seizures.

1.6.3 Anticonvulsant Treatment

The current clinical management for hypoglycemia is the intravenous administration of 25 – 50% dextrose in saline. However, this may result in increased excitability and glucose reperfusion injury (Suh et al, 2007b). In addition, continued seizing will place a substantial energy demand on the neurons involved in these seizures. Concomitantly, other energy demanding processes such as neuronal death and glial infiltration to the site of injury are occurring (Walling et al, 2007). Infusion of NMDA and AMPA receptor antagonists after hypoglycemia reduced the damage to the rodent neocortex and hippocampus (Nellgard and Wieloch, 1992). As such, it can be postulated that the use of anticonvulsants during hypoglycemia may aid in ameliorating the depletion of energy substrates and reducing neuronal damage.

1.6.4 EEG: Cortex, Hippocampus, Brainstem

While previous studies have investigated the EEGs in hypoglycemic animals, these were not performed in the context of a diabetic model (del Campo et al, 2009; Velsek et al, 2008). Also, the hippocampus has been shown to generate ictal activity in vitro (Abdelmalik et al, 2007) but in vivo studies suggest that site of origin may be in deeper brain layers. Penfield and Jasper were the first to propose, in the 1950s, the idea that seizures can originate in subcortical regions of the brain that subsequently spread to the cortex. Since then, other studies have demonstrated the role of the thalamus in generating spike-wave discharges and the importance of the brainstem and cerebellum in various types of seizures (Norden and Blumenfeld, 2002).

The SNR has been shown to control seizures through the administration of GABA agonists and that this region mediates seizures in deep brain layers such as superior colliculus and pontine reticular formation (Moshe et al, 1995). Therefore, it was hypothesized that seizures originate from deeper brain structures or the spinal cord. Additionally, tonic-clonic seizures and loss of righting reflex are observed in animals during hypoglycemia (Velsek et al, 2008). Seizures with such manifestation are known to originate in the brainstem region (Browning, 1985).
Chapter 2

OBJECTIVES

2.1 Main Objective

The main objective of this thesis is to establish a novel rat model of IDDM in young animals and describe hypoglycemic seizures both behaviourally and in terms of blood glucose threshold. This model is then used to measure cortical, hippocampal and brainstem EEGs, observe their progression during hypoglycemia, and effectiveness in predicting seizures.

2.2 Specific Hypothesis

The general aims of the study have been divided into specific hypotheses

1) Insulin induced hypoglycaemia results in seizures in juvenile diabetic rats. In clinical studies, it has been demonstrated that children are more prone to developing seizures (Davis et al., 1997).

2) Severity of hypoglycemic seizures is associated with acute mortality. The “dead in bed syndrome” occurs 3 times more frequently in young people with diabetes than those without (Heller, 2002). While cardiac arrhythmias have been thought to be the cause, hypoglycemic seizures provide another potential explanation (Tattersall and Gill, 1991).

3) Anticonvulsant treatment will mitigate seizures and reduce mortality. Prophylactic treatment with anticonvulsants will prevent the development of seizures. Anticonvulsant treatment will reduce excitability and therefore prevent the adverse outcomes of hypoglycemic seizures.

4) EEG activity will predict seizure development, mortality and detect electrographic discharges in cortical, hippocampal and brainstem regions. Clinical studies have reported that aberrant EEGs at the time of the initial diagnosis of IDDM can be predictive of subsequent convulsions (Tupola et al., 1998). EEG abnormalities have been observed during moderate and severe hypoglycemia in adult non-diabetic animals (Auer, 2004; del Campo et al., 2009). In addition, the substantial nigra, pedunculopontine tegmental
nucleus and superior colliculus were shown to be involved in the control of hypoglycemic seizures (Velsek et al, 2008), suggesting that these originate in deeper brain structures.

5) Hypoglycemic seizures are associated with neuronal damage. Studies have shown that the cortex as well as the CA1 and dentate gyrus regions of the hippocampus are particularly vulnerable to hypoglycemic damage while the brainstem and cerebellum are resistant (Auer, 1989). In addition, diabetes and seizures have been reported to exacerbate this damage (Bree et al, 2009). However, the distribution of damage in juveniles has not been assessed.
Chapter 3
MATERIALS AND METHODS

3.1 Animals

All studies were done in accordance with and approved by the Animal Research Council at the University Health Network (Toronto, Ontario, Canada). Male Sprague Dawley rats from Charles River Laboratories (21-day-old, weaned), weighing 40-60 grams were housed in pairs in a temperature-controlled environment with ad libitum access to water, a standard rat chow diet and under a 12-hour light/dark cycle. The rats were ear-punched for identification.

3.2 Induction of Diabetes

Streptozotocin (STZ) was dissolved in 0.1mM sodium citrate (Fisher Scientific), buffered to a pH of 4.5 with 1M citric acid (Fisher Scientific), to make a 10 mg/ml solution just prior to injection. The 22-day-old rats (40-60g) were fasted overnight (14-16 hrs) and received IP injections of one of the following doses of STZ: 60mg/kg, 75mg/kg or 80mg/kg, to induce diabetes. Controls were randomly selected and injected with the 0.1mM sodium citrate buffer vehicle. Following STZ or citric buffer vehicle injections, body weights and tail vein blood glucose levels (BG) using a Hemocue Glucose 201 glucometer (Hemocue, Vitaid) were measured. Measurements were made 2 days after STZ IP and every 4 days subsequently to confirm stable diabetes.

3.3 Induction of Hypoglycemia

Animals were fasted overnight (14-16 hours), administered insulin IP (15units/kg; Humulin R; Eli Lilly and Company) IP and video-monitored for 5 hours to detect motor seizures or convulsive seizure-like events, these are referred to as “seizures” for the rest of this thesis. Fasting BG was measured (see methods above) prior to insulin IP and hourly leading up to seizures. “Lowest BG” was considered as the minimum blood sugar measured in the time period leading up to seizures or up to 3 hours in animals that did not seize. In some cases this measure was taken 15-30 mins prior, as blood samples were difficult to acquire when rats were lethargic. In a subset of animals (25 diabetics and 16 controls), BG was measured at onset of seizure before
administering treatment to determine the blood glucose threshold for seizures. After seizures, rats that appeared healthy as defined by resumption of normal grooming habits as well as eating and drinking, survived. Rats that had continuous seizures despite treatment and were not responsive were euthanized and their brains were collected for histological analysis. These animals were categorized as non-surviving with seizures and mortality (S+M) in all further analyses.

3.4 Treatment of Seizures

The following treatment strategies were employed to treat or attenuate seizures:

Group 1: 1 g/kg glucose at seizure onset with each subsequent seizure treated with 0.5g/kg of glucose.

Group 2: Diazepam (Sandoz Canada Inc., 5 mg/kg), Phenytoin (Sandoz Canada Inc., 50 mg/kg) and 1 g/kg of glucose at seizure onset with each subsequent seizure treated with diazepam (2.5 mg/kg).

Group 3: Diazepam (5 mg/kg), Phenytoin (50 mg/kg) and 1 g/kg of glucose at seizure onset with each subsequent seizure or blood glucose < 2.5mM (measured every 30 mins after seizure) treated with 0.5g/kg of glucose.

Group 4: Prophylactic treatment at the time of insulin administration with Diazepam (5 mg/kg), Phenytoin (50 mg/kg) and 1 g/kg glucose at seizure onset with subsequent seizures treated with 0.5g/kg of glucose.

3.5 Seizure Scoring

A seizure score was developed to characterize and quantify the SLEs observed in this model (Table 1). The behaviours in this score chart were based on previous scores (Veliskova, 2006) and combined according to our observations. Treatment was administered to rodents who displayed obvious twitches (seizure score ≥ 2.5).
**Table 1**: Seizure score chart to characterize and quantify the observed seizures

<table>
<thead>
<tr>
<th>Score</th>
<th>Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Head up or tail stiff</td>
</tr>
<tr>
<td>1</td>
<td>Rearing and Falling</td>
</tr>
<tr>
<td>1.5</td>
<td>Myoclonic jerk or hindlimbs/forelimbs stretched</td>
</tr>
<tr>
<td>2.0</td>
<td>Body extended with hindlimb or forelimb digging or body curled</td>
</tr>
<tr>
<td>2.5</td>
<td>Unilateral forelimb or hindlimb clonus</td>
</tr>
<tr>
<td>3</td>
<td>Ipsilateral or contralateral forelimb and hindlimb clonus</td>
</tr>
<tr>
<td>3.5</td>
<td>Bilateral forelimb or hindlimb clonus</td>
</tr>
<tr>
<td>4</td>
<td>Wild running</td>
</tr>
<tr>
<td>4.5</td>
<td>All limbs clonus</td>
</tr>
<tr>
<td>5</td>
<td>Head bent backwards forelimb clonus with tonus of hindlimbs</td>
</tr>
<tr>
<td>5.5</td>
<td>Partial barrel roll</td>
</tr>
<tr>
<td>6</td>
<td>Full barrel roll</td>
</tr>
<tr>
<td>7</td>
<td>Full tonic extension</td>
</tr>
</tbody>
</table>

The seizure score that was obtained in 5-minute epochs was used for further analysis. Animals were also grouped by the types of seizures that were observed. Two types of categorization were performed. Firstly, rats were segregated by whether or not the brainstem was recruited during the seizure (seizure score ≥ 4.0). Secondly, rats were also placed in three categories (1) Partial seizures: only involving one limb (seizure score=2.5); (2) Partial to Secondary generalization: the seizure spreads from one limb to other areas (3) Secondary generalization: without prior evident partial seizures (seizure score ≥ 3.0).

### 3.6 Statistical Analyses

All statistical tests were performed with Sigma Stat software (11th version; Systat Software Inc.). Comparison of data between two groups was carried out via Students’ T-test. To
compare proportions of the various treatment groups, a Chi-squared or Fisher’s exact test was used. Significance was set at P<0.05. All error bars indicate ± SEM.

3.7 EEG Monitoring

13 diabetic rats were implanted with intracranial electrodes using the methodology previously described (del Campo et al, 2009). Data acquisition and analysis were conducted using pCLAMP 9.0 (Molecular Devices, Inc., California U.S.). Two animals had electrodes in the left motor cortex and right hippocampal formation (CA1) and 11 were implanted in the right hippocampal (CA1) and left mesencephalic reticular formation. Animals were allowed 5-7 days recovery prior to hypoglycemic insult and EEG recording. Continuous EEG recordings were obtained one hour prior to insulin administration to establish a baseline (after overnight fast). Rodents were then recorded for up to 5 hours after insulin IP or until seizure occurred. Simultaneous video monitoring was performed on all animals.

3.8 Preliminary Histology

Rats were transcardially perfused with 100 mL 0.1% Phosphate Buffered Saline (PBS) and 40 mL 4% paraformaldehyde and the whole brains were removed at either two or seven days post-hypoglycemic insult. Whole brains were cut into 3mm sections and embedded in paraffin. Paraffin blocks were sliced 10 microns in thickness every 50 microns with a total of 10 slices taken from each block. Fluorojade C (emission: 450nm, excitation 530nm) staining was performed to mark degenerating neurons. Staining methods were performed in accordance with the manufacturer’s protocol (millipore.com) and cells that showed fluorescence were counted as having undergone neurodegeneration.
Chapter 4
RESULTS

4.1 Optimal STZ Dosage for Induction of Diabetes

Diabetes was confirmed by measuring blood glucose (BG) from a tail vein blood sample 2 days after STZ was administered to 22-day-old male rats. Hyperglycemia (Non-fasting BG > 11.1mM) was used as the inclusion criterion for diabetes. The dosage of 60mg/kg failed to induce diabetes in any animal (n=16). A 50% success rate was observed when the dose was increased to 75mg/kg (n=28). 80mg/kg of STZ was 91% successful in inducing stable diabetes in rats (n=44) (p<0.001; Figure 1A). This dosage was selected for future experiments.

Over the course of seven days after STZ administration, in addition to hyperglycemia, the diabetic animals had a significantly decreased weight gain (p<0.005). The average body weight at the end of this time was 78.7±1.2 g (n=40) compared to age-matched control animals; 100.5±2.5 g (n=17) (Figure 1B). In addition, rats were monitored for 60 days after STZ administration to ensure that the diabetic state was not reversible. In addition to continued hyperglycemia, the rats where STZ administration was successful reached a weight range lower than controls (Figure 1C).
**Figure 1:** Effects of different STZ dosages on the diabetes induction and weight gain of juvenile rats

**A:** STZ dose of 75mg/kg induced diabetes in 50% of rats (n=14/28) whereas with a dose of 80mg/kg, diabetes resulted in 91% of rats (n=40/44); (p<0.001). (*) A statistically significant difference

**B:** Rats treated with 80mg/kg STZ and became diabetic displayed significantly decreased weight gain (*) after 1 week compared to controls (p<0.005) and non-diabetics (STZ-treated rats where diabetes did not occur)

**C:** Weight increase over 60 days. At PN22 animals had similar weights. After STZ (80mg/kg) or citric buffer injections, both controls (CB) and non-diabetic rats gained weight faster than diabetic (STZ) rats over time
4.2 Incidence of Hypoglycemia, Seizures and Mortality

To assess the prevalence of seizures during hypoglycemia and concomitant effects on diabetic (STZ) rats, postnatal (PN) day 28-30 rats (n=69) that had been diabetic for 5-7 days, were rendered hypoglycemic with the acute administration of insulin (15units/kg, IP). Animals were fasted overnight prior to receiving insulin, since this reliably induces hypoglycemic seizures (Velisek et al, 2008). Rats were then administered insulin and video monitored for 4-5 hours. Hypoglycemia was confirmed through BG levels measured from a tail vein sample.

Both citric buffer administered control (CB) and diabetic (STZ) rats attained hypoglycemia (blood glucose < 3.5 mM). Incidence of hypoglycemia was 68% (n=43/63) in STZ and 86% (n=19/23) in CB rats (Figure 2A). Concomitantly, behavioural seizures were observed in the majority of hypoglycemic animals. 86% (n=37/43) of STZ and 100% (n=19/19) of CB rats displayed convulsions (Figure 2B). Mortality resulted in 35% (n=13/37) of STZ and 42% (n=8/19) of CB rats that exhibited seizures. Conversely, 100% survival occurred in STZ (n=26; p<0.005) and CB (n=4) animals that did not seize (Figure 2C). 6 of the 26 surviving rats that did not seize also reached hypoglycemic levels. Consequently, there is a high incidence of seizures during hypoglycemia and these seizures are a necessary precondition for mortality.
**Figure 2:** Incidence of hypoglycemia, seizures and mortality after insulin IP (15u/kg) in overnight-fasted PN 28-30 rats; 1 week after STZ (diabetic rats) or CB (controls)

**A:** Incidence of hypoglycemia in all animals injected with insulin; 68% of STZ (n=43/63) and 83% of CB (n=19/23); no statistically significant difference between both groups

**B:** Incidence of seizures in rodents where hypoglycemia was confirmed (BG<3.5mM); 86% of STZ (n=37/43) and 100% of CB (n=19/19); no significant difference exists between the two groups

**C:** Rate of mortality after insulin administration and hypoglycemia; 35% (n=13/37) of STZ and 42% (n=8/19) of CB rats. 0% mortality observed in non-seizing STZ (n=0/26) and CB (n=0/4) rodents. (*) Denotes a statistically significant difference in survival between seizing and non-seizing rats in the STZ group (p<0.005)
4.3 Blood Glucose Decrease within the First Hour is Predictive of Seizure but not Mortality

Blood glucose (BG) levels were measured prior to insulin administration and every subsequent hour until seizure onset. The average latency to seizures was significantly faster in CB (1.1±0.1 hours) than in STZ rats (2.4±0.2 hours, p<0.001). Prior to insulin administration, BG levels in CB were significantly lower than in STZ rodents (p<0.001; Figure 3A/B). Fasting BG levels prior to the injection of insulin were not a significant factor, in CB (Figure 3A) or STZ rats (Figure 3B), for whether or not these animals displayed seizures. Concurrently, incidence of mortality was not significantly affected by fasting pre-insulin BG levels in CB (Figure 3C) or STZ rats (Figure 3D).

After insulin administration, both STZ and CB rats that eventually developed seizures had a significant drop in BG levels within the first hour compared to the NS groups (BG in CB; NS: 6.2±0.9 mM, CB with seizure: 2.0±0.1 mM, STZ; NS: 9.5±1.6 mM, STZ with seizure: 3.8±0.3 mM, Figure 3E) (p<0.001).

In the STZ group, the 37 rats displaying seizures (lowest BG: 2.0±0.1 mM), reached a significantly lower (p<0.002) blood glucose level than the 6 non-seizing (NS) animals (BG: 2.8±0.3 mM); there was no significant difference in lowest BG measured between those that survived or died (S+S: 2.0±0.1 mM; S+M: 1.9±0.1 S+M, Figure 3F). Control NS rodents did not achieve hypoglycemic levels. These data indicate that there is a BG range or threshold in which seizures occur as demonstrated in the next section.
Figure 3: Relationship of blood glucose (BG) decrease after insulin IP (15u/kg) to seizure and survival

A: No significant difference in BG of CB rats with or without seizures at 0hr (time of insulin administration). At 1 hr, BG in non-seizure (NS) group was significantly higher (*) than the seizure + survival (S+S); (p < 0.001)

B: No significant difference in BG of STZ rats with or without seizures at 0hr (time of insulin administration). BG in NS group was significantly higher, at 1 hr (*) and 2hr (**) post-insulin, than the S+S group; (p < 0.001)

C: No significant difference in BG of CB rats at 0hr or 1hr post-insulin in S+S and S+M groups

D: No significant difference in BG of STZ rats at 0hr, 1hr or 2hr post-insulin in S+S and S+M groups

E: BG levels at 1 hr post-insulin is significantly greater in CB; NS: 6.2±0.9mM than CB with seizure: 2.0±0.1mM (*), and in STZ; NS: 9.5±1.6 mM compared with STZ with seizure: 3.8±0.3 mM (**); (p<0.001)

F: Lowest BG measured is significantly higher in the NS group (n=6): 2.8±0.3 mM compared with either S+S (n=24): 2.0±0.1 mM or S+M (n=13): 1.9±0.1 mM groups (*); (p<0.002)

Table 2: Mean blood glucose levels in STZ rats, measured hourly, related to outcome (±S.E.M)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>0 hr</th>
<th>N</th>
<th>1 hr</th>
<th>N</th>
<th>2hr</th>
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<td>NS</td>
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<td>S+M</td>
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<td>3.2 ± 0.4</td>
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<tr>
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<td>26</td>
<td>4.1 ± 0.4</td>
<td>17</td>
<td>3.1 ± 0.5</td>
</tr>
</tbody>
</table>
4.4 Blood glucose Threshold for Seizures is Similar in STZ and CB Rodents

To establish the BG level for seizure threshold, comparing STZ and CB animals, BG at seizure onset (defined as a seizure score ≥2.5, refer to Table 1) was measured in 41 rats (25 STZ and 16 CB rats). As mentioned above, prior to insulin administration, BG levels in CB (6.3±0.3 mM) were significantly lower than in STZ rats (12.4±1.3 mM; p<0.01) (Figure 4A). Despite the increased initial BG, there was no significant difference in the BG measured at seizure onset in STZ (1.8±0.2 mM) compared to CB rats (1.6±0.1 mM) (Figure 4A). In addition, the average rates of BG decrease in STZ (4.4±0.36 mM/hr) and CB rats (4.5±0.5 mM/hr) were similar. As previously stated, the average latency to seizures was significantly faster in CB (1.1±0.1 hours) than in STZ (2.4±0.2 hours) rats (p<0.001). The two-fold higher initial BG in STZ rats can explain the difference in seizure latency between the two groups. These data suggest that there exists a common blood glucose threshold at which seizures will occur whether or not the animal is diabetic.

In addition, BG at seizure onset was not predictive of whether rats displayed a single seizure (that was controlled by the administration of glucose) or subsequent seizures (after treatment). In CB rats, the BG levels were 1.7±0.2 mM and 1.6±0.1 mM in groups with single and multiple seizures, respectively. Likewise, in diabetics, BG levels at seizure onset of rats with a single seizure and multiple seizures were 1.9±0.2 mM (n=10) and 1.9±0.3 mM (n=12), respectively (Figure 4B).

At seizure onset, no significant difference was measured in the BG levels of STZ surviving (1.8±0.2 mM, n=15) and non-surviving rats (2.2±0.3 mM, n=5). Similarly, the BG of CB rats did not differ significantly between those that survived (BG 1.7±0.1 mM, n=9) and those that did not (BG 1.5±0.1 mM, n=5, Figure 4C).
**Figure 4:** Blood glucose (BG) threshold for seizures in CB vs. STZ rats and association with survival and seizures after treatment

**A.** Initial BG (prior to insulin IP) is significantly lower in CB (6.3±0.3 mM; n=16) than in STZ rats (12.4±1.3 mM; n=25) (p<0.01). BG at seizure onset (observed seizure score ≥2.5) is similar in CB (1.6±0.1 mM) and STZ rats (1.8±0.2 mM)

**B:** No significant association between BG levels at seizure onset and post-treatment seizures. BG levels glucose at seizure onset for CB: 1 seizure; 1.7 ±0.2 mM and >1 seizure (glucose was unable to mitigate treatment); 1.6±0.1 mM and STZ: 1 seizure; 1.9±0.2 mM (n=10), >1 seizure; 1.9±0.3 mM (n=12) are not statistically different

**C:** No significant difference between BG levels at seizure onset comparing survival and mortality. BG for CB: S+S; 1.7±0.1 mM (n=11) and S+M; 1.5 ±0.1 mM (n=5); STZ: S+S; 1.8±0.2 mM (n=17) and S+M; 2.2±0.32 mM (n=5) are not significantly different
4.5 Progressions and Characterization of Seizures

In order to establish a seizure model, the observed seizure behaviours must first be described. Reliable video recordings were obtained in 22 STZ and 16 CB rats that displayed seizures. All rats were treated with glucose at seizure onset. Seizure scoring was performed to quantify the severity of the observed seizures (Table 1). Prior to seizure-inducing hypoglycemic levels, all rats that reached moderate hypoglycemia (<3.5 mM) became lethargic. In the absence of EEG recording, it was difficult to discern less severe seizure-like activity (seizure score: 0.5-2) from lethargic behavior. Therefore, only rats that reached a seizure score of ≥2.5 were treated and classified as having seized (see methods).

The rats’ behaviours were scored and the highest seizure score (0.5-7) attained within each 5-minute epoch was plotted to represent the development of seizures over time. Both CB (Figure 5A, B) and STZ rats (Figure 5C, D) exhibited varying progression of seizure severity, with some rats undergoing slow progression from mild to more severe seizures over 30 minutes or longer whereas others immediately showed severe seizures. With the exception of 2 rats, CB; S+S rats, displayed seizures that evolved quickly. This trend was not evident in the STZ; S+S group. Notably, mortality occurred in all rats that reached a seizure score of 7.

As it was difficult to observe trends in seizure progression due to variability, animals were grouped by whether or not the brainstem was recruited during the seizures. This was set at seizure scores ≥ 4.0 where the righting reflex was lost (Veliskova, 2006). The loss of righting reflex is observed in 73% of CB: S+S animals (n=8/11) and 100% of CB: S+M animals (n=5/5) as well as 53% of STZ: S+S animals (n=9/17) and 100% of S+M rats (n=5/5).

To further relate seizures to observations in the epilepsy patients, the seizures were segregated (see methods) into a) partial seizures (≤2.5), b) partial seizures with secondary generalization, and c) secondary generalization without prior evident partial seizures (≥3). STZ and CB animals were categorized accordingly with the following results. Partial seizures: CB: S+S: 10% (n=1/11), CB: S+M: 0%, STZ: S+S: 30% (n=5/17), STZ: S+M: 0%. Secondary generalization: CB: S+S: 45% (n=5/11), CB: S+M: 40% (n=2/5), STZ: S+S: 35% (n=6/17), STZ: S+M: 80% (n=4/5). Generalized: CB: S+S: 45% (n=5/11), CB: S+M: 60% (n=3/5), STZ: S+S: 35% (n=6/17) and STZ: S+D: 20% (n=1/5).
Figure 5: Evolution of seizures over the course of hypoglycemia (each trace represents a different animal)

A: CB; S+S; n=11

B: CB; S+M; n=5

C: STZ; S+S; n=17

D: STZ; S+M; n=5
4.6 Continued Seizures are Associated with Mortality

While it is evident that hypoglycemia and the resulting seizures are associated with mortality, it is not clear whether hypoglycemia was the sole cause. (Figure 4C). Therefore, the analysis of seizure scores was used to isolate the effects of seizures on mortality. The severity of the first seizure, prior to administration of glucose, was not significantly different between CB rats: S+S: 4.3±0.5 (n=11) and S+M: 6.0±0.4 (n=5) as well as STZ rats: S+S: 3.4±0.3 (n=17) and S+M: 4.7±0.7 (n=5); (Figure 6A).

The most severe seizure, measured by the maximum seizure score, suffered by CB rats was also similar in S+S: 4.9±0.4 (n=11) and S+M rats: 6.5±0.3 (n=5). Likewise, STZ rats: S+S: 4.3±0.4 (n=17) and S+M: 5.5±0.4 (n=5) illustrated the same trend (Figure 6B).

Nevertheless, the number of seizures an animal underwent was a stronger predictor of mortality. The number of seizures observed was significantly lower in the CB rats: S+S: 1.6±0.3 (n=11) compared with S+M: 7.8±2.7 (n=5); p<0.01. This difference was also observed between STZ rats: S+S: 1.6±0.2 (n=17) and S+M: 4.4±1.2 (n=5); p<0.001 (Figure 6C). These data confirms that continued seizures that are unresponsive to glucose, possibly due to impaired glucose control (Velsek et al, 2008), are correlated with acute mortality in this model.
**Figure 6:** Comparing the association between seizure severity and mortality

**A:** No significant difference in the mean score of the first seizure treated in CB rats: S+S: 4.3±0.5 (n=11) and S+M: 6.0±0.4 (n=5) and STZ rats: S+S: 3.4±0.3 (n=17) and S+M: 4.7±0.7 (n=5)

**B:** No significant difference in the mean maximum seizure score observed in CB rats: S+S: 4.9±0.4 (n=11) and S+M: 6.5±0.3 and STZ rats: S+S: 4.3±0.4 (n=17) and S+M: 5.5±0.4 (n=5)

**C:** A statistically significant difference in the mean number of seizures between (*) CB rats: S+S: 1.6±0.3 (n=11) and S+M: 7.8±2.7 (n=5; p<0.01) and between (*) STZ rats: S+S: 1.6±0.2 (n=17) and S+M: 4.4±1.2 (n=5; p<0.001)
4.7 Anticonvulsants Reduce Seizure Incidence

To evaluate the efficacy of glucose, the current treatment strategy, and whether outcome differed with anticonvulsant treatment, the following strategies were employed on STZ rats:

Group 1 (glu): Glucose (1g/kg) at first seizure onset with subsequent seizures treated with glucose (0.5g/kg).

Group 2 (ac+1xglu): Diazepam (5mg/kg), Phenytoin (50mg/kg) and glucose (1g/kg) at seizure onset with subsequent seizures treated with Diazepam (2.5 mg/kg).

Group 3 (ac+multiple glu): Diazepam (5mg/kg), Phenytoin (50mg/kg) and glucose (1g/kg) at seizure onset with subsequent seizures or hypoglycemia (BG<2.5 mM), treated with 0.5g/kg of glucose.

The initial trend in BG decrease after insulin was similar in all 3 groups, indicating that the BG decline was not a confounding factor of treatment outcome (Figure 7A). Treatment with glucose at seizure onset was not always successful in mitigating seizures. In group 1, 55% (n=12/22) of animals exhibited seizures after treatment compared with 24% of animals in group 2 treated with glucose plus anticonvulsants (n=5/21) (Figure 7B). Anticonvulsants followed by repeated glucose administration at BG<2.5mM, significantly reduced the incidence of post-treatment seizures compared to glucose alone with only 15% of rodents (n=2/14; group 3) undergoing subsequent seizures (p<0.02) (Figure 7B). Additionally, the amelioration of seizures in 85% of group 3 rats was not significantly different from the 76 % in treatment group 2.

Despite the success of anticonvulsant treatment in mitigating seizures, with multiple glucose administration, there was no impact on survival, 93% (n=1/14; group 3), whereas with glucose administration alone, the survival was higher (77%, n=17/22; group 1) (Figure 7C). As the anticonvulsants appeared to be masking motor convulsions despite continued severe hypoglycemia, the survival rate of 93% (n=13/14; group 3), with anticonvulsants and multiple doses of glucose, was significantly higher (p<0.02) than with a single glucose administration (48%, n=11/21;group 2) (Figure 7C).
**Figure 7:** Efficacy of treatments, Group 1-3, in preventing subsequent seizures and mortality in STZ rats. Glu: Glucose (1g/kg) at seizure onset with each subsequent seizure treated with glucose (0.5g/kg). ac+1xglu: Diazepam (5mg/kg), Phenytoin (50mg/kg) and glucose (1g/kg) at seizure onset with subsequent seizures treated with Diazepam (2.5 mg/kg). ac+multiple glu: Diazepam (5mg/kg), Phenytoin (50mg/kg) and glucose (1g/kg) at seizure onset with subsequent seizures or hypoglycemia <2.5 mM, treated with 0.5g/kg of glucose

**A:** BG decrease is not significantly different in seizing animals regardless of treatment; seizure+ survival+ glucose treatment (S+S Glu), seizure + survival + anticonvulsant treatment (S+S AC), seizure + mortality + glucose treatment (S+M Glu) seizure + mortality + anticonvulsant treatment (S+M AC); see Table 3.

**B:** No significant difference in the incidence of seizures post-treatment in Group 2; 24% (n=5/21) compared with Group 1; 55% (n=12/22). (*) Significantly lower incidence of seizures post-treatment in Group 3; 15% (n=2/14) compared with Group 1; 55% (n=12/22) (p<0.02)

**C:** (*) Significantly higher survival rate in Group 3; 93% (n=1/14) compared with Group 2; 48% (n=10/21) (p<0.02). No significant difference between treatment multiple glu, with; Group 3: 93% (n=1/14) or without anticonvulsants; Group 1: 77% (n=17/22)
Table 3: Mean blood glucose level measured hourly related to treatment and survival

**S+S Glu**: Seizure + Survival; glucose treated

**S+S ACs**: Seizure + Survival; glucose and anticonvulsant treated

**S+M Glu**: Seizure + Mortality; glucose treated

**S+M ACs**: Seizure + Mortality; glucose and anticonvulsant treated

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<th>n</th>
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<th>n</th>
<th>1 hr</th>
<th>n</th>
<th>2hr</th>
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<td>10</td>
<td>14.3 ± 2.3</td>
<td>9</td>
<td>3.6 ± 0.5</td>
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<tr>
<td><strong>S+S Glu</strong></td>
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<td>12.5 ± 1.6</td>
<td>17</td>
<td>4.3 ± 0.5</td>
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<td>3.2 ± 0.6</td>
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<tr>
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<tr>
<td><strong>S+M Glu</strong></td>
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<td>14.7 ± 3.2</td>
<td>5</td>
<td>3.5 ± 0.2</td>
<td>3</td>
<td>1.7 ± 0.2</td>
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</tbody>
</table>
4.8 Anticonvulsants Reduce the Number of Seizures

As with CB and glucose-treated STZ rats there was no significant difference in the severity of the first seizure prior to treatment. The rats in Group 2: S+S: 3.6±0.6 (n=8) and S+M: 5.4±0.7 (n=8) or in Group 3: S+S: 3.2±0.3 (n=13) and S+M: 2.5 (n=1) demonstrated no association between the initial seizure score and mortality (Figure 8A).

Interestingly, the maximum seizure score in Group 2 was significantly higher in S+M rats: 6.1±0.6 (n=8) when compared with S+S rats: 4.0±0.6 (n=8) (p<0.01). In contrast, the maximum score observed in the glucose-treated Group 1 rats that survived (4.3±0.4; n=17) was not significantly different to those that died (5.5±0.4; n=5). This was also the case in Group 3 rats: S+S: 4.0±0.4 (n=13) and S+M: 5.5 (n=1), though the sample size of the latter was not adequate (Figure 8B).

As previously reported, glucose-treated rats demonstrated an association between the number of seizures and mortality (S+S: 1.6±0.2; n=17 compared with S+M: 4.4±1.2; n=5) (p<0.001). However, rats in Group 2 (S+S: 1.3±0.2; n=8 and S+M: 1.4±0.2; n=8) and Group 3 (S+S: 1.8±0.2; n=13 and S+M: 3.0; n=1) exhibited no such relationship. Additionally, anticonvulsant-treated animals in Group 2 where mortality resulted, underwent a significantly lower number of seizures than those that were glucose treated; p<0.05 (Figure 8C). This phenomenon is similar to results in Figure 7 where anticonvulsants suppress motor convulsions while animals may continue to undergo non-convulsive seizures. As such, glucose must be replenished to mitigate these seizures and prevent mortality.
Figure 8: Effects of treatments on the seizure scores and mortality in STZ rats. glu: Glucose (1g/kg) at seizure onset with each subsequent seizure treated with glucose (0.5g/kg). ac+1xglu: Diazepam (5mg/kg), Phenytoin (50mg/kg) and glucose (1g/kg) at seizure onset with subsequent seizures treated with Diazepam (2.5 mg/kg). ac+multiple glu: Diazepam (5mg/kg), Phenytoin (50mg/kg) and glucose (1g/kg) at seizure onset with subsequent seizures or hypoglycemia <2.5 mM, treated with 0.5g/kg of glucose

A: No significant difference in the mean score of first seizure attained in glu rats: S+S: 3.4±0.3 (n=17) and S+M: 4.7±0.7 (n=5); ac+1xglu rats: S+S: 3.6±0.6 (n=8) and S+M: 5.4±0.7 (n=8); or ac+multiple glu rats: S+S: 3.2±0.3 (n=13) and S+M: 2.5 (n=1)

B: Mean maximum seizure score attained in glu rats: S+S: 4.3±0.4 (n=17) and S+M: 5.5±0.4 (n=5); (*) ac+1xglu rats: S+S: 4.0±0.6 (n=8) and S+M: 6.1±0.6 (n=8) (p<0.01); or ac+multiple glu rats: S+S: 4.0±0.4 (n=13) and S+M: 5.5 (n=1)

C: Mean number of seizures in (*) glu rats: S+S: 1.6±0.2 (n=17) and S+M: 4.4±1.2 (n=5) (p<0.001); ac+1xglu rats: S+S: 1.3±0.2 (n=8) and S+M: 1.4±0.2 (n=8); or ac+multiple glu rats: S+S: 1.8±0.2 (n=13) and S+M: 3.0 (n=1). (**) Significant difference also exists between glu rats: S+M and ac+1xglu rats: S+M (p<0.05)
Prophylactic Treatment Increases Seizure Latency

Given that treatment at seizure onset significantly reduced the incidence of subsequent seizures, a fourth treatment strategy was employed to test whether prophylactic treatment with anticonvulsants at the time of insulin administration would prevent seizures. The protocol for group 4 was prophylactic treatment at the time of insulin administration with Diazepam (5 mg/kg), Phenytoin (50 mg/kg), followed by glucose (1g/kg) at seizure onset with subsequent seizures treated with glucose (0.5g/kg).

Prophylactic treatment with anticonvulsants did not significantly reduce the proportion of rats that became hypoglycemic. 46% (n=6/13) of group 4 rats reached hypoglycemic levels compared with 68% (n=43/63) of rats previously reported in Figure 2A (Figure 9A). Surprisingly, 83% (n=5/6) of rodents exhibited seizures; a non-significant decrease compared with the 86% (n=37/43) that seized after insulin administration alone (Figure 9B). While 100% (n=5/5) of the group 4 rats seized and survived, this does not constitute a significant reduction from the 65% (n=24/37) that survived when treated at seizure onset (Figure 9C).

BG at seizure-onset in prophylactically treated animals (n=5; 1.5±0.4 mM) was not statistically lower than in untreated animals (n=25; 1.8±0.2mM), perhaps due to high variability in these BG values (Figure 9D). Interestingly, the latency to seizure onset was significantly increased in prophylactically treated animals compared to those not prophylactically treated: 3.3±0.2 hours and 2.4±0.1 hours, respectively (Figure 9E; P<0.01).
**Figure 9:** Efficacy of prophylactic treatment, Diazepam (5 mg/kg), Phenytoin (50 mg/kg), followed by glucose (1g/kg) at seizure onset with subsequent seizures treated with glucose (0.5g/kg), on seizure incidence, outcome, threshold and latency in STZ rats

**A:** Incidence of hypoglycemia (BG<3.5mM) in; 46% of prophylactically treated rats (n=6/13) and 68% of rats not prophylactically treated (n=43/63); no statistically significant difference between both groups

**B:** Incidence of seizures in rodents where hypoglycemia was confirmed; 83% of prophylactically treated rats (n=5/6) and 86% of rats not prophylactically treated (n=37/43); no statistically significant difference between both groups

**C:** Rate of mortality after seizures; 0% (n=0/5) of prophylactically treated rats and 35% (n=24/37) of rats not prophylactically treated; no statistically significant difference between both groups

**D:** BG at seizure onset with prophylactic treatment: treated animals; 1.5±0.4 mM (n=5) and untreated animals; 1.8±0.2 mM (n=25)

**E:** Prophylactic treatment increases seizure latency (*): Prophylactic (n=5): 3.3±0.2 hrs, treatment at onset + survived (n=24): 2.3±0.2 hrs, treatment at onset + died (n=13): 2.4±0.2 hrs (p<0.05)
4.10 Electroencephalogram During Hypoglycemia

EEG recordings were obtained in 13 STZ rats. While nine of these rats became hypoglycemic, only five displayed behavioral seizures. Unfortunately, to avoid interrupting the recordings, consistent monitoring of BG was not possible. Previous studies have established that EEG suppression and isoelectricity is associated with severe hypoglycemia (Auer 2004). The trace of a hypoglycemic animal in Figure 10 demonstrate an obvious reduction in EEG amplitude, in the hippocampus (CA1) and brainstem (MRF), 3.5 hours after insulin administration and approximately 30 minutes prior to a seizure. The lowest blood glucose measured in this animal was 2.6 mM at three hours post-insulin. This phenomenon was observed in only two of the hypoglycemic animals with only one proceeding to convulsive seizures. Hence, this EEG suppression is not predictive of a subsequent seizure. The traces from baseline to 3 hours after insulin (Figure 10A/B) are representative of recordings in the other seven hypoglycemic rodents. These animals displayed slowing of EEG waves relative to the baseline between 2-3 hours after receiving insulin (Figure 10A/B).
**Figure 10:** Comparison of EEG changes over the period of hypoglycemia, relative to baseline in hippocampus (traces 1-4) and brainstem (traces 5-8)

**A:** Representative EEG traces of hippocampus (CA1) at baseline (post-fasting; prior to insulin IP), 2 hours, 3 hours (slower waves) and 3.5 (EEG suppression) hours after insulin IP

**B:** Representative EEG traces of mesencephalic reticular formation (MRF) at baseline (post-fasting; prior to insulin IP), 2 hours, 3 hours (slower waves) and 3.5 (EEG suppression) hours after insulin IP
4.11 Electrographic Seizure Activity is Not Associated with Convulsive Behaviour

In this model, seizures occurred independently of EEG suppression since only 20% (n=1/5) of the rodents showed reduced EEG amplitude prior to seizing. Brief electrographic seizures were recorded in the CA1 (Figure 11A, bottom trace) region in one animal and both the CA1 as well as brainstem (Figure 11C) in another. Though rats at this stage of hypoglycemia were lethargic and immobile, no overt convulsive behaviour was observed. The rat exhibiting the non-convulsive hippocampal seizure (Figure 11A) had received treatment (Diazepam, Phenytoin and glucose) for an antecedent seizure without an electrographic correlate (Figure 11B). Notably, there was no corresponding electrographic activity in the animal’s contralateral cortical recording (Figure 11A, top trace).

Electrographic seizures were similarly observed in the mesencephalic reticular formation of the brainstem (Figure 11C). Figure 11D, again, illustrates motion artifact induced by seizure-like behavior, but no rhythmic or paroxysmal change in the EEG immediately before, or during the seizure-like behaviour. Consistent with previous studies (del Campo et al, 2009), the behavioural manifestations classified, as “seizures” were not associated with any characteristic seizure patterns in EEG recordings. However, the possibility, that movement artifact during convulsions are masking electrographic seizures, cannot be excluded.
**Figure 11:** Electrographic seizures, recorded in the hippocampus (CA1) and brainstem (MRF), are not associated with convulsive behaviour

**A:** Electrographic seizure activity observed in CA1 (lower trace) after suppression of EEG activity. No ictal activity in the contralateral cortex (upper trace)

**B:** EEG of the rat in A during a behavioural seizure; no ictal activity is observed

**C:** EEG recording of hippocampus and contralateral MRF obtained in diabetic rat during hypoglycemia. Electrographic seizure activity observed after suppression of EEG activity. Lower trace illustrates magnification of the area in the gray box

**D:** EEG of the rat in C during a behavioural seizure; no ictal activity is observed
Chapter 5

DISCUSSION

The objective of this thesis was two-fold: 1) to establish a model of Insulin Dependent Diabetes Mellitus (IDDM; juvenile diabetes) in young rats and, (2) to utilize this model to characterize behavioural manifestations of seizures and assess their outcome. It is important to conduct these studies in juveniles as this population is more susceptible to neuronal excitability and seizures (Moshe et al, 1995; Veliskova et al, 2004; Rakhade and Jensen, 2009) and the latency to seizure generalization is shorter (Veliskova, 2006). These seizure effects have not been specifically studied in the context of diabetes and hypoglycemia. Diabetic patients also appear to have 10 times greater risk of sudden unexplained deaths or the “dead-in-bed” syndrome (Secrest et. al, 2010), potentially as the result of seizures (Tattersall and Gill, 1991). It is necessary to study hypoglycemic seizures within the diabetic paradigm, as diabetic patients are especially susceptible to hypoglycemic episodes due to exogenous insulin treatment (Cryer, 2004).

5.1 Optimal STZ Dosage for Induction of Diabetes

While the STZ model of diabetes induction has been well established in adult animals (Like and Rossini, 1976), a model in young animals has not been established or characterized. Although, STZ administration does not trigger the same inflammatory reaction in the pancreas as seen in spontaneous diabetic rats (BB rats), this spontaneous form of diabetes results only in older animals. In the BB rat, hyperglycemia, weight loss and other symptoms of diabetes develop at around 12 weeks of age (Rees and Alcolado, 2005). However, STZ is sufficient to induce hyperglycemia and weight loss in juvenile animals.

While in adult animals, 40-60mg/kg STZ is sufficient to induce diabetes (Szkudelski et al, 2001); we determined that young animals required the higher dose of 80 mg/kg. Lower dosages (60 mg/kg and 75 mg/kg) were unsuccessful. Younger animals required a more potent dose as their pancreatic β-cells or islet cells may be more resilient to the damage induced by the drug. The confirmation of the hormone levels of glucagon and insulin may have provided
further evidence for the diabetic condition in these animals. However, as the animals were hyperglycemic and insulin administration was able to lower the BG levels, this was used as evidence that the animals had developed in IDDM. Future experiments, particularly with an insulin-treated model of diabetes, would require that other parameters such as insulin and glucagon be measured.

Another characteristic of IDDM is the slowed growth in the rats where STZ was effective, demonstrated by the significantly impaired weight gain (Gan et al, 2012). This is due to the breakdown in fat and muscle to compensate for the inability to uptake glucose through GLUT4 transporters for energy (Brown and Ransom, 2007). The animals where STZ was not successful in inducing diabetes (non-diabetic) did not demonstrate this impairment (Figure 1B). In fact, over 60 days, the growth in non-diabetics was similar to age-matched controls (Figure 1C). Therefore, there were no long-term toxicity effects of STZ per se. Conversely, diabetic animals had diminished weight gain over the course of 60 days. Therefore, the dose of 80mg/kg was determined as appropriate for the induction of a stable model of juvenile diabetes in young rats.

5.2 Incidence of Hypoglycemia, Seizures and Mortality in Controls Compared to Diabetic Animals

Hypoglycemia was induced in 68% (43/63) of diabetic rats and 86% (19/23) of controls injected with insulin. The lower incidence of hypoglycemia in the diabetic rodents can be explained by the higher fasting BG levels in this group (Figure 3A/B, Figure 4A). There was a strong association between hypoglycemia and seizures in both diabetic and control rats. The majority of hypoglycemic rats, 37 of 43 diabetics and 19 of 19 controls, displayed seizures. Curiously, control animals had a higher incidence of seizures than diabetic animals even though hypoglycemic seizures are rare in the non-diabetic population. In non-diabetic individuals, endogenous insulin is subject to normal homeostatic mechanisms such as the attenuation of insulin release and the increase in glucagon and adrenaline (Cryer, 2004). Exogenous insulin, used to treat diabetes, is not subject to these processes that act to correct hypoglycemia. However, control rats received exogenous insulin which would similarly impair these mechanisms. It can be postulated that the shift in equilibrium between the exogenous insulin and
endogenous glucagon as well as epinephrine eventually leads to seizures (Cryer 2004). In addition, if the animals are subject to an insulin treatment protocol this may result in impaired counterregulation due to more frequent moderate hypoglycemic episodes.

While several clinical studies have addressed hypoglycemic seizures, to my knowledge, the incidence of seizures in hypoglycemia is not definitively known (Tupola et al, 2004; Howarka et al, 2000; Lapenta et al, 2010; Davis et al, 1997; Kaufmann et al, 1999). In many cases, seizures may be misdiagnosed since they present similarities to other autonomic responses during hypoglycemia (Lapenta et al, 2010). As such, the observed incidence of seizures in these rodents cannot be correlated to the human population. It should also be noted the experiments were performed exclusively in male animals to avoid the confounding effects of hormone changes. As the development of the proconvulsant region in the SNR appears to be mediated by testosterone, the seizure incidence in females may be lower (Veliskova et al, 2004).

The high rates of seizures could also be attributed to fasting as this has been demonstrated to downregulate hyperpolarizing KATP channels thus increasing seizure susceptibility (Velsek et al, 2008; Ghasemi et al, 2010). Though these experiments were performed in non-diabetic, adult rodents, this downregulation may explain why 75% of hypoglycemic seizures in children are nocturnal (Buckingham et al, 2008).

5.3 Blood Glucose Decrease is a Predictor of Seizures

All diabetic animals had similar fasting blood glucose levels, whether or not seizures eventually developed. Likewise, the controls also had related fasting blood glucose levels; whether or not there was a progression to seizures, but their average fasting BG levels were about ½ of the fasting diabetic BG levels. In our model, the drop in blood glucose within the first hour post-insulin administration was predictive of whether animals progressed to hypoglycemia and subsequent seizures in both diabetics and controls. Diabetic animals that did not seize presented with significantly higher glucose levels (9.5±1.6mM) 1 hr after insulin administration than animals that had seizures (3.8±0.3mM). As well, in controls, the BG measure of 6.2±0.9mM dropped significantly to 2.0±0.1mM (Figure 3E). This rate of BG decline was not an indicator of mortality as BG measurements were similar in surviving and non-surviving animals (Figure 3C/D).
While 6 of the animals that did not seize were hypoglycemic, their lowest BG levels (2.8±0.1mM) were significantly higher than those that had seizures (2.0±0.3mM) (Figure 3F). These data provide evidence for a blood glucose threshold at which seizures occur. It should be noted that in some cases, obtaining sufficient blood from the tail vein of lethargic animals was difficult. As blood glucose measurements were taken hourly, the values in Figure 3 represent blood glucose levels up to 30 minutes prior to seizures in some cases as seizure latency was 2.4±0.2 hours in diabetic rats and 1.1±0.1 hours in controls. As such, these measures were not obtained precisely at seizure onset and do not provide information for the BG threshold for seizures.

5.4 Seizure Threshold is Similar in Diabetic and Control Animals

To establish the threshold BG for seizures, measure of BG levels at seizure onset was required. Seizures occurred at similar blood glucose levels in diabetic (1.8±0.2mM) and control (1.6±0.1mM) rodents. While it was expected that diabetic animals would have increased seizure susceptibility, (Ghasemi et al, 2010) this result may be explained by the short period of diabetes (5 to 7 days). Recordings of the CA3 indicated increased spontaneous activity in rats that were hypoglycemic for 4 weeks and not 3 weeks or less (Margineanu et al, 1998).

The BG measured at seizure onset in the animals was not representative of the levels observed in hypoglycemic children where severe hypoglycemia resulting in seizures and coma is approximately 3.3-3.9mM. However, these animals were subject to an acute hypoglycemic episode and instead multiple hypoglycemic episodes may cause animals to seize at higher BG levels. Recurrent hypoglycemic episodes occur more often in diabetic patients and therefore the resulting impaired counterregulation may lower seizure threshold. Future experiments will evaluate the impact of recurrent hypoglycemic seizures, as this is more representative of clinical observations. While in vivo studies have reported that repeated moderate hypoglycemia is neuroprotective (Puente et al, 2010), effects of inducing recurrent hypoglycemic seizures may lead to increased spontaneous excitability similar to kindling. This is supported by the data in Figure 6C where animals can have multiple seizures that are unresponsive to glucose, also shown by Velsek et al (2008) in nondiabetic adult animals.
5.5 Behavioural Manifestations of Seizures

Development and progression of convulsive behaviour was quite variable in the diabetic and control animals. In control S+S animals, it was observed that seizures progressed quickly and were ameliorated by treatment. Control S+M animals or diabetic animals did not demonstrate this trend (Figure 5A-D). To better understand these seizures, animals were categorized into seizure types (see methods) as observed in epileptic patients (Berg et al, 2010).

The rats that underwent slow seizure progression fell into two general patterns. Firstly, partial seizures, defined by their slow progression and isolated limb twitching, were rare in controls (1/10; 10%) and more frequent in diabetic animals (5/17; 30%) that survived. Secondly, in some rats, seizures evolved to recruit other limbs and the loss of righting reflex. Interestingly, in diabetic rats that died, seizures did not remain focal as 80% (4/5) demonstrated secondary generalization and one animal showed a generalized seizure without prior partial seizures. Similarly in the controls, 3/5 had partial seizures followed by secondary generalization, and the other two displayed a generalized seizure. It is unclear how the generalization of seizures relates to hypoglycemia as BG was not measured at each seizure and further experimentation with BG measures at the onset of every seizure would be required to address this question.

The young age of the rats offers a possible explanation for the high incidence of seizures becoming generalized and the short time period in which this occurs (Veliskova, 2006). Due to low sample size in the groups where mortality resulted, it is unclear whether the observed trends can be related to mortality.

5.6 Severity of Seizures and Mortality

Rates of mortality are similar in diabetic (35%) and control animals (42%). Though 100% survival was observed in non-seizing rodents, it is not sufficient evidence for whether seizures directly cause mortality especially since BG in non-seizing hypoglycemic animals was significantly higher than in those that seized (Figure 3F). Nevertheless, blood glucose at seizure onset was similar in diabetic and control animals that survived or died and therefore was not a factor for mortality (Figure 4B). This is not surprising, as the first seizure score prior to
treatment when blood glucose levels were measured was similar in these four groups (Figure 6A). This may be due to low sample size of the animals that died (n=5).

The observed generalized tonic-clonic seizures suggest involvement of brainstem regions (Browning, 1985). In the animal models of seizures, brainstem involvement is defined by the loss of righting reflex where animals can no longer hold themselves up (Veliskova, 2006). This occurred at a seizure score of 4 or higher, when all limbs began twitching. This behaviour was always observed in animals that died; controls (5/5) and diabetic rats (5/5). This difference was not statistically different from control (8/11; 73%) and diabetic animals (9/17; 53%) that survived. The involvement of the brainstem in seizures can cause mortality as this region controls autonomic functions such the heart rate and breathing. Disruption in the functioning of this region can cause cardiac and respiratory changes that lead to mortality (Moseley et al, 2012).

Though the maximum seizure score attained was similar in controls and diabetic animals regardless of survival (Figure 6B), this was only an assessment of one seizure event. On the other hand, the number of seizures was found to be significantly higher in animals that died in either diabetic or control groups (Figure 6C), confirming a strong association between seizures and mortality. Impaired glucose control may be a potential cause of these continued seizures (Velsek et al, 2008). Since the purpose of the experiments was to establish the BG at the first seizure onset, measures were not taken at subsequent seizures. Future experiments will include BG measurements at subsequent seizures to confirm if BG threshold for seizures has been lowered or whether other mechanisms are involved in these continued seizures.

5.7 Anticonvulsant Treatment in Diabetic Animals Reduces Seizure Incidence while Prophylactic Treatment Delays Onset

The correction of hypoglycemia with multiple glucose administration in many cases was required to prevent mortality. In 12/22 animals, a single dose of glucose was not sufficient to ameliorate seizures (Figure 7B). Animals still required more glucose after subsequent seizures in order to correct hypoglycemia and stop the convulsions. However, this was not always successful in attenuating seizures and preventing mortality. Four animals still continued seizing despite multiple glucose treatments, with mortality as the end result (Figure 7C).
Anticonvulsant treatment at seizure onset was successful in mitigating motor convulsions in only 7/35 (20%) animals seizing post-treatment (Figure 7B). However, without continued glucose replenishment, mortality occurred in 11/21 rats despite anticonvulsant treatment (Figure 7C). It can be posited that anticonvulsants only stop the behavioural manifestations of seizures. Lack of energy substrate may cause continued non-convulsive seizures. However, with the absence of EEG recordings in this group, subclinical seizures could not be detected. Further support for this can be provided by the significantly decreased number of apparent motor seizures in anticonvulsant-treated rats (1.4±0.2) compared with glucose-treated rats (4.4±1.2) that died (Figure 8C). Rats treated with anticonvulsants and not repeated glucose administration did not display intermediate seizure scores, but rather progressed to severe tonic extensions (Score=7) (Figure 8B). As evidenced in Figure 11B and D, it is possible to have electrographic seizures in the absence of a behavioural correlate (del Campo et al, 2009; Velsek et al; 2008). Continued monitoring of BG after treatment with anticonvulsants revealed that these animals displayed fluctuations in glucose levels and as such survival was improved with the correction of hypoglycemia in conjunction with anticonvulsants (Figure 7C).

Though anticonvulsants mitigated seizures when administered at seizure onset, a few animals still had seizures. In the case of one rat, seizures were uncontrollable despite continued glucose administration implying that other mechanisms are involved beyond glucose deprivation (Suh et al, 2007b). Diazepam a GABA agonist and Phenytoin that acts on sodium channels are only able to transiently stop seizures and without glucose replenishment the seizures become more severe (Figure 8B). These data suggest the involvement of enhanced NMDA activity in these seizures (Suh et al, 2007b, Veliskova et al, 2007).

As anticonvulsant treatment mitigated motor seizures, animals were treated prophylactically to determine whether seizure onset could be prevented. In prophylactically treated animals, the seizure incidence of 83% (5/6) was comparable to the 86% (37/43) observed in animals treated at onset. In addition, while mortality was abolished in this group (0/5), this difference was not statistically significant. Despite the administration of anticonvulsants, seizures eventually occur unless the energy substrate is administered. Anticonvulsants did significantly delay the seizure latency. This finding may have clinical relevance as administering anticonvulsants to hypoglycemic patients may prevent continued seizures that exacerbate
hypoglycemia-induced neuronal damage (Bree et al 2009) while blood glucose is being brought back to euglycemic levels.

5.8 Electroencephalogram Recordings During Hypoglycemia and Seizures

As observed in previous studies in adult rodents (del Campo et al 2009), our EEG data show that behavioral seizures in juvenile diabetic animals do not originate in the hippocampus. There were no apparent predictive EEG changes seen in the 5 of 9 hypoglycemic rats that proceeded to seize. Severe hypoglycemia (≤1.0mM) as indicated by EEG isoelectricity (Auer et al, 1984) was not a requirement for convulsions; 4 of 5 rodents had behavioral seizures without suppression of EEG. There was slowing in the EEG waves prior to seizures in these animals, indicative of less severe hypoglycemia (Auer, 2004). Spectral analysis of EEG waves will be required to accurately quantify these changes. It is not surprising that EEG isoelectricity is not correlated as the associated BG of ≤1.0 mM was not a requirement for seizures. This was confirmed in Figure 4A where the mean BG at seizure onset was 1.6±0.1 mM in controls and 1.8±0.2 mM in diabetic rats.

Conversely, electrographic seizures recorded in the hippocampus (+/-brainstem) were brief (10-30s), failed to spread to the cortex, and occurred in the context of EEG suppression that is associated with neuronal damage (Auer 2004). These observations suggest that the same mechanisms responsible for neurotoxicity may be crucial in sustaining the depolarization of susceptible neuronal networks, but the lack of adequate energy substrate could prevent spread to higher brain regions. In addition, the observed seizure-like behavior (some of which could be interpreted as primitive locomotion phenomenology) as seen in decerebrate preparations (Whelan 1996) may suggest a brainstem or spinal cord generator. Further studies recording EEG activity from other brainstem and spinal regions are required to test this postulation.
5.9 Hypoglycemic Seizures are Associated with Minimal Hippocampal and Cortical Damage

Preliminary histological evaluation showed minimal damage in the hippocampus and cortex (Figure 12). Consistent with previous literature, neuronal degeneration was negligible, as BG was not maintained at severely hypoglycemic levels \( \leq 1.0 \) mM (Auer 2004). Our results demonstrate that seizures occur at higher threshold BG levels, \( \sim 1.5-2.0 \) mM. Even then, the damage was clearly reversible as no damage was seen in rats sacrificed 7 days after seizures. Interestingly, there was a trend to more damage in diabetic animals when compared with controls that also underwent seizures. However, these results would have to be compared against control and diabetic rats that did not undergo hypoglycemia and seizures. It has been previously reported that damage in adult diabetic animals only occurs after severe prolonged hypoglycemia (Bree et al, 2009). The continued increase in intracellular calcium due to activation of ionotropic glutamate receptors (Somjen, 2004) as well as the increase in ROS may mediate this neuronal damage (Suh et al, 2007b). The distribution of neuronal damage, localized to the hippocampal and cortical regions, may be related to the distribution of astrocytes and glycogen (Brown and Ransom, 2007) and future experiments may be required to determine this.

There are several factors that potentially explain the mechanisms for seizures during hypoglycemia. The downregulation of K\(_{\text{ATP}}\) channels that impair potassium buffering have been postulated to enhance excitability (Velsek et al, 2008). The decrease in intracellular ATP impairs the activity of the Na\(^+\)/K\(^+\)-ATPase and thus the cell’s ability to restore intracellular potassium and extracellular sodium levels leading to an enhanced glutamate receptor function (Marinelli et al, 2001). This is further exacerbated by the increase in excitatory amino acids such as glutamate and aspartate as well as ammonia (Auer, 2004) that has been correlated with the onset of seizures (Lewis et al, 1974). This increase in production of glutamate and aspartate is the result of accumulating oxaloacetate from an impaired Kreb’s cycle (Auer, 2004). Additionally, this increase in extracellular glutamate can cause swelling of astrocytes particularly in the cortex and hippocampus (Han et al, 2004). The ability of AP7, a glutamate receptor antagonist, to significantly mitigate hypoglycemic motor seizures is evidence for the crucial role of EAAs in these convulsions (Veliskova et al, 2007). The increased excitability and decreased
inhibition can cause synchrony of neuronal firing, which can be propagated by electrical conduction through gap junctions (Somjen, 2004).

### 5.10 Conclusions and Future Experiments

In summary, a novel model of juvenile diabetes in young rats has been established. As well, the following can be concluded from the evaluation of this model. (1) Hypoglycemic seizures due to exogenous insulin administration can occur in juvenile animals, independent of diabetes. (2) The decline in blood glucose during the first hour following insulin administration is predictive of seizures. (3) A single hypoglycemic event results in a similar blood glucose seizure threshold level in diabetic and control animals. Future experiments performed in juvenile animals with recurrent episodes of hypoglycemia can test the hypothesis that seizure susceptibility would be increased. In addition, these experiments would need to be performed in an insulin-treated model of diabetes, which is more clinically relevant. (4) Seizures have varied progression with the possible involvement of the brainstem. (5) Seizures are a necessary precondition for mortality in this juvenile diabetic hypoglycemia model. (6) Continued repeated seizures rather than the blood glucose threshold predict mortality. (7) Anticonvulsant treatment, prophylactically and at seizure onset, attenuates the motor component of seizure and increases seizure latency, but is not sufficient to prevent seizures and mortality. (8) Electrographic seizures can be recorded in the hippocampus and MRF without behavioural correlates.

While preliminary histological experiments show minimal damage in the cortex and hippocampal regions, further quantification of these samples are required. This damage would need to be compared against diabetic and control animals that were not made hypoglycemic. Since the BG threshold for seizures is higher than the observed BG levels causing damage $\leq 1.0$ mM (Auer, 2004) more sensitive measures of outcome should be considered. Reduced LTP after hypoglycemic seizures have been demonstrated in young non-diabetic rats (Yamada et al, 2004) prior to neuronal damage and can be evaluated in diabetic animals post-seizure. In addition, GFAP staining can be employed to assess astrocytic damage, as astrocytic swelling may be a consequence of the increased glutamate release in hypoglycemia (Han et al, 2004).

Furthermore, spectral and wavelet analysis of EEGs may elucidate trends that predict the onset of seizures. As well, EEGs recorded a few days after hypoglycemia can be utilized as
another assessment of outcome. Finally, since seizures may be generated from lower brain regions such as the medulla oblongata or spinal cord (Velsek et al), EEG recordings of these regions can confirm this hypothesis.

While the findings of the above experiments have evaluated the damaging effects of iatrogenic hypoglycemia, such as uncontrolled seizures leading to mortality, it must be noted that these effects do not outweigh the benefits of insulin as a treatment for IDDM. Nevertheless, these results not only reinforce the need for monitoring blood glucose and insulin levels but for understanding of the underlying mechanisms and brain regions involved in generating hypoglycemic seizures. This knowledge may aid in mitigating such detrimental outcomes of treatment and improving the patient’s quality of life. Thus concludes the body of my thesis. Below is a list of references and preliminary histological experiments.


Preliminary Experiments

Fluorojade staining was performed to visualize degenerating neurons and assess the effects of hypoglycemic seizures on the brain tissue of diabetic and control animals. None of the diabetic rats at one week after hypoglycemic insult showed fluorojade positive (+) cells (n=7). However, 48 hours after the hypoglycemic episode, damage (fluorescing cells) in hippocampus and cortex was observed in 50% of the diabetic rats (n=8), only two of which showed numerous (>10 cells) fluorojade (+) cells, suggesting much damage to these regions, while minimal (<3 cells) damage was observed in the other two rats (Table 4). Control rats (n=3), dissected 48 hours after hypoglycemic seizures, demonstrated either minimal (<3 cells; cortex) or no fluorojade (+) cells. This damage in the cortex was not localized to a specific area (Figure 9).

Table 4: Hypoglycemic seizures are not clearly associated with histological damage.

<table>
<thead>
<tr>
<th>Type (D/C)</th>
<th>Time after Seizure (days)</th>
<th>Hippocampus (# of cell)</th>
<th>Cortex (# of cells)</th>
<th>Initial BG</th>
<th>BG at seizure</th>
<th>Seizure Score</th>
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<td>1.0</td>
<td>5</td>
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<tr>
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<td>1</td>
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<td>1.6</td>
<td>7</td>
</tr>
<tr>
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<tr>
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Figure 12: Minimal neuronal damage as measured by fluorojade positive (+) cells in the hippocampus of an STZ rat 48 hrs after hypoglycemic seizures (white arrows: left); Magnification (40X). Enlarged image of a fluorojade (+) neuron (right).

Blood glucose at seizure onset: 0.9 mM; maximum seizure score: 2.5.