Behavioral and Electrographic Abnormalities due to Repeated Hypoglycemic Episodes in Mice

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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Abstract

Severe hypoglycemia poses the greatest challenge to glycemic control in diabetic patients, especially children with type 1 diabetes mellitus. Although in vivo animal models exist for investigating the effects of hypoglycemia, few studies examine repeated hypoglycemia and none investigate within the context of a juvenile animal model. The main objective of this thesis was to examine electroencephalographic (EEG) and behavioral abnormalities manifesting as a result of repeated hypoglycemia in juvenile diabetic and non-diabetic mouse models. Using a novel implantation technique, the hippocampal and cortical EEG were recorded during repeated insulin-induced hypoglycemia. It was discovered that repeated hypoglycemia exacerbated behavioral convolution severity and promoted epileptiform EEG activity within the hippocampus and cortex of both diabetic and non-diabetic animals. Furthermore, sustained hypoglycemia caused a significant decrease in hippocampal EEG activity in diabetic animals compared with non-diabetics. These results suggest recurrent hypoglycemia may promote and worsen seizures associated with hypoglycemia in diabetic children.
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Chapter 1
Introduction

1.1. Energy Metabolism in the Brain

1.1.1. Metabolic Demand of the Brain

The human brain is metabolically one of the most active organs in the body, representing only 2% of total weight in adult humans, yet responsible for 20% of the resting body’s $O_2$ consumption and receiving 15% of total cardiac output (Clarke and Sokoloff, 1999; MacDonald and King, 2007). As measured in a young adult man, the rate of cerebral $O_2$ consumption was 49mL $O_2$ per minute (Sokoloff, 1960), which was estimated to support the steady turnover of 7mmol (approx. $4 \cdot 10^{21}$ molecules) of adenosine triphosphate (ATP) per minute (Clarke and Sokoloff, 1999). Virtually all brain $O_2$ is used in the oxidation of carbohydrates, the brain’s primary energy substrate (Sokoloff, 1960).

In contrast to most tissues, which can use carbohydrates as an alternative fuel to lipid-derived substrates (Cryer, 1997), the brain appears restricted almost exclusively to glucose for its energy requirements. In 1931, Lennox measured the $O_2$ and $CO_2$ content in arterial and internal jugular venous blood in 120 human subjects, reporting an average cerebral respiratory quotient of 0.95 (metabolism of carbohydrates correspond to 1.00 while that of protein is about 0.82 and fat is near 0.70; Ganong, 2001) and providing the first support of the important role of carbohydrates in brain energetics. Brain glucose utilization has been measured at 31$\mu$mol/100g per minute (Clarke and Sokoloff, 1999). The high energy demands of the brain, combined with its limited glycogen reserves and inability to synthesize glucose, result in an absolute requirement for
continuous cerebral circulation to supply O$_2$ and glucose to maintain proper brain functionality (Auer, 2004; Clarke and Sokoloff, 1999; Cryer et al., 2003; Isaev et al., 2007; Suh et al., 2007).

1.1.1. Sources of Glucose

For humans, glucose is obtained from three sources: from the external environment through ingestion; from glycogen reserves through glycogenolysis; and from various precursors, such as lactate, amino acids, and glycerol, through gluconeogenesis (Cryer, 1997).

Ingested carbohydrates from the external environment are broken down, through mechanical digestion and by salivary amylase, into short polysaccharides and disaccharides within the oral cavity. In lower regions of the gastrointestinal tract, enzymes from the pancreas and intestinal glands further degrade simple sugars into their component monosaccharide units. Glucose, among other monosaccharides, is then absorbed through the intestinal epithelium and enters circulation via the hepatic portal vein (Silverthorn, 2000).

Glycogen is a large, branched polysaccharide that functions as a storage form of glucose in animal cells. The major sites of glycogen in the body are the liver and skeletal muscle (10% and 2% by organ weight, respectively. Berg et. al, 2002). Total glycogen that can be mobilized averages about 390mmol (Nilsson, 1973), which alone could maintain euglycemia for up to 4 hours (Cryer, 1997). The breakdown and storage of glycogen requires glycogen phosphorylase and glycogen synthase, respectively (Cryer, 1997). Glycogen metabolism is accomplished by (1) releasing glucose-1-phosphate from glycogen, which can be used in the glycolytic pathway; (2) converting glucose-1-phosphate to glucose-6-phosphate, which can be converted directly to
glucose and released into circulation; and (3) remodeling of glycogen to allow for processing by the pentose phosphate pathway, yielding reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ribose derivatives (Berg et. al, 2002). Although glycogenolysis produces glucose in the liver, glycogen metabolism in muscle is done via glycolysis, which produces pyruvate. Pyruvate can be reduced to lactate and then transported to the liver, where it serves as a precursor for gluconeogenesis (known as the Cori cycle; Cryer, 1997; Frier and Fisher, 2007).

The brain, itself, contains a limited store of glycogen. Öz and colleagues (2007) used in vivo localized $^{13}$C NMR spectroscopy in 9 human subjects to report brain glycogen content at ~3.5µmol/g. Clarke and Sokoloff (1999) predicted glycogen reserves would be depleted in less than five minutes if used as the brain’s sole energy source.

Gluconeogenesis involves the conversion of pyruvate-derived substrates to glucose (essentially, “reverse glycolysis”), which requires key enzymes including pyruvate carboxylase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase and fructose-1,6-bisphosphatase. These enzymes are highly expressed in the liver and kidney but not muscle, fat, or brain tissues (Cryer, 1997). After an overnight fast, glycogenolysis and gluconeogenesis each account for about half of total glucose production (Tayek and Katz, 1996). However, after prolonged fasting (24-48 hours), gluconeogenesis becomes the sole source of glucose for the body (Rothman et al., 1991).

Regardless of its source, circulating glucose enters the brain by crossing the blood-brain barrier (BBB), assisted by uncoupled glucose transporters (GLUTs) that facilitate diffusion. The human genome contains 14 members of the GLUT family (SLC2A gene), each of which shows
remarkable tissue-specificity (for a review of GLUT family expression profile, see Scheepers et al., 2004). With reference to the brain, the predominant GLUT isoforms are GLUT1 and GLUT3. GLUT1 has near ubiquitous expression across cell types and is the primary transporter located on the luminal and adluminal surfaces of microvessel endothelium lining the BBB (Simpson et al., 2007; Vannucci et al., 1997). Furthermore, GLUT1 expression is negatively regulated by glucose concentration (Clarke and Sokoloff, 1999). GLUT3 functions as the principal neuronal glucose transporter (Vannucci et al., 1997).

**1.1.2. Aerobic Glucose Catabolism**

Cellular catabolism of glucose commences within the cytosol, beginning with the phosphorylation of glucose, by hexokinase, to glucose-6-phosphate. Glucose-6-phosphate will then enter the Embden-Meyerhoff glycolytic pathway, ultimately being cleaved into two pyruvate molecules and yielding a net of two equivalents of both ATP and NADPH. Tissue $O_2$ levels and the energy status of the cell dictate the fate of pyruvate: it can either (1) enter the mitochondria for conversion to acetyl CoA, (2) be reduced to lactate within the cytosol, or (3) undergo transamination. In aerobic conditions, the majority (85%; Clarke and Sokoloff, 1999) of pyruvate will follow the first pathway (Bryne and Roberts, 2004; Dwyer, 2002).

Within the mitochondrial matrix, pyruvate is decarboxylated and combines with coenzyme A to produce acetyl CoA. Acetyl CoA will enter the tricarboxylic acid (TCA) cycle, an eight-step reaction cycle. The end product of the TCA cycle is oxaloacetate, which can combine with new incoming acetyl CoA to repeat the cycle. For each molecule of glucose, the TCA cycle produces 6 NADH, 2 FADH$_2$, 2 GTP, and 4 CO$_2$ molecules (Bryne and Roberts, 2004; Dwyer, 2002).
In the final step of aerobic glucose catabolism, the cofactors NADH and FADH$_2$, which were generated during glycolysis and the TCA cycle, translocate to the inner mitochondrial membrane and transfer their high energy potential electrons to O$_2$ via the electron transport chain (ETC). The ETC is a group of complex carrier molecules which couple the transfer of high energy potential electrons to ATP generation via oxidative phosphorylation. The ETC is responsible for the bulk of ATPs (32 out of 36) generated during complete oxidative catabolism of one glucose molecule (Bryne and Roberts, 2004; Dwyer, 2002).

In summary, contrasting with the generation of 2mol ATP per mole of glucose in anaerobic metabolism, each mole of glucose undergoing oxidative metabolism theoretically yields 36mol of ATP (Magistretti et al., 2000).

1.1.3. Alternate Sources of Energy

Under normal conditions, glucose metabolism fulfills all nutritional needs of the brain (Seisjö, 1978). Indeed, other potential energy substrates are either inadequately supplied to the brain or enzymes required for their metabolism are not present in sufficient amounts in the brain (such as those for glycerol and ethanol; Clarke and Sokoloff, 1999) to provide a substantial energy source. Nonetheless, in situations of glucose depletion, the brain can temporarily utilize ketone bodies (in particular, acetoacetate and β-hydroxybutyrate) for its energy requirements.

Ketone bodies are byproducts of fatty acid metabolism, and are primarily released from the liver into circulation (Sokoloff, 1973). The pathways of ketone utilization in the brain are similar between acetoacetate and β-hydroxybutyrate. Acetoacetate is converted to acetyl CoA by 3-
ketoacid CoA transferase and acetyl CoA thiolase, before subsequently being incorporated into the TCA cycle. β-hydroxybutyrate requires 3-hydroxybutyrate dehydrogenase to convert it to acetoacetate before undergoing the same enzymatic cascade (Sokoloff, 1973).

Normally, levels of circulating ketone bodies are low, however, during prolonged fasting, lipolysis and ketogenesis accelerate and cause an increase in their concentrations. Hasselbalch and colleagues (1995), for example, found in 9 human subjects that a 3.5-day fast resulted in an increase of arterial β-hydroxybutyrate concentration from 0.23 mM to 2.94 mM. Furthermore, BBB permeability for β-hydroxybutyrate increased from 0.008 to 0.101 µmol/g·min. Similar increases have also been documented in acetoacetate (Owen et al., 1967). After long-term fasting (5-6 weeks), ketone bodies were shown to account for a majority (~70%) of metabolic substrate utilized by the brain (Owen et al., 1969).

1.2. Neuropathophysiology of Glucose Deprivation

1.2.1. Glucose Concentrations in Blood and Cerebrospinal Fluid

As previously discussed, the brain is dependent upon a steady supply of glucose from systemic circulation to maintain neuronal activity. Blood glucose levels are maintained by balancing the secretion of glucose-mobilizing hormones, such as glucagon, epinephrine, and glucocorticoids, with the release of glucose-storing hormones, such as insulin (Silverthorn, 2000). This regulatory system keeps blood glucose levels tightly maintained: measurement of human arterial plasma glucose concentration over a 24-hour period was averaged to approximately 5 mM (Gerich, 1993), remaining above 3 mM during fasting periods (Consoli et al., 1987), and rising to a maximum of ~9 mM postprandial (Rizza et al., 1980). Blood-to-brain glucose transport is a
function of cerebral arterial glucose concentration, and is not directly influenced by any of the glucoregulatory hormones (Cryer et al, 1997; Greutter et al., 1998). Glucose levels in the cerebrospinal fluid (CSF) fluctuate depending on the body metabolic state, however a general rule is CSF glucose content should be two-thirds that of a simultaneously withdrawn blood plasma sample (Merritt, 1937).

1.2.2. Effect of Glucose Deprivation on Neuronal Metabolism

Reduction in circulating glucose levels result in a decreased rate of glucose transport to the brain. In situations where blood glucose levels fall below 2mM, brain glucose consumption exceeds the transport capacity of the BBB, and CSF glucose levels approach 0mM (Choi et al., 2001).

A drop in extracellular glucose availability contributes to decreased activity in the Ebden-Meyerhoff glycolytic pathway and TCA cycle. Indeed, two previous studies in rodents have shown insulin-induced hypoglycemia was associated with a decrease in pyruvate and all TCA cycle intermediates except oxaloacetate, which increased (Goldberg et al., 1966; Lewis et al., 1974). It is possible that oxaloacetate accumulates because there is a decrease in acetyl CoA amounts, with which it usually condenses to form citrate and repeat the TCA cycle (Auer, 2004; Fig. 1).

Oxaloacetate is also a substrate for aspartate aminotransferase, which converts it to the excitatory amino acid (EAA) aspartate. The buildup of oxaloacetate during glucose deprivation drives an increase in aspartate production, according to Le Châtelier’s Principle (Auer, 2004). One study demonstrated that levels of aspartic acid increased 1600% to ~5mM in the extracellular matrix of
the brain during iodoacetate-induced inhibition of glucose metabolism (Sandberg et al., 1985). Increases in aspartate concentrations secondarily drive the conversion of α-ketoglutarate to glutamate, another EAA (Fig. 1, Auer, 2004).

Glucose deprivation also affects oxidation-reduction systems in cells. Normally, glucose catabolism leads to the generation of NADH, but with reduced glycolytic flux during glucose deprivation, evidence has been presented that there is a fall in the NADH/NAD⁺ ratio (Lewis et al., 1974). Other oxidation-reduction systems, including NADPH/NADP⁺ and glutathione/glutathione disulfide, are similarly affected by reduced glucose levels (Auer, 2004).

Reduction in glucose has been shown to cause a rise in tissue pH. In 1981, Pelligrino et al. demonstrated that insulin-induced hypoglycemia in rats is accompanied by an increase in pH of 0.04-0.05 units (CSF) and 0.15 units (intracellular). Increased ammonia production from protein deamination as well as reductions in lactate generation are thought to account for this observation (Auer et al., 2004).
Figure 1. Alterations in glucose oxidative metabolism in the brain due to hypoglycemia. Relative thickness of arrows represents quantitative flow of metabolites along the pathways (Auer RN. 2004. Hypoglycemic brain damage. Forensic Sci Int. 146(2-3): 105-10. © by Elsevier Inc. Adaptation by permission of copyright holder).

1.2.3. Neurodegeneration due to Glucose Deprivation

Glucose deprivation will impact the ability of neurons to generate ATP. The main energy-demanding function of the brain is sustaining transmembrane ion gradients and facilitating ion fluxes related to neural excitation and conduction (Du et al., 2008), hence a decrease in ATP will likely affect these functions first. Indeed, the loss of electrolyte homeostasis is suggested by a study from Weiloch al. (1984), which demonstrated insulin-induced hypoglycemia in rats
resulted in a drop of extracellular Ca\(^{2+}\) from 1.17±0.14mM to 0.18±0.28mM while extracellular K\(^{+}\) rose from 3.4±0.94mM to 48±12mM.

Decreases in extracellular Ca\(^{2+}\), and consequent rise in intracellular Ca\(^{2+}\), has been hypothesized to initiate brain damage (Seisjö, 1981) in a process coined “excitotoxicity” (Olney, 1974). Work by Olney and colleagues (1986) suggested EAA-related neurotoxicity was a direct consequence of excessive neuronal excitation. The influx of Ca\(^{2+}\) to the cytosol promotes exocytosis of EAAs from presynaptic terminals. Increased EAA release, coupled with the disruption of ATP-dependent EAA reuptake mechanisms in neurons and glia, causes sustained high extracellular concentrations of aspartate and glutamate (Agardh et al., 1981).

Extracellular EAAs have previously been shown to interact with both NMDA and non-NMDA receptors, which opens nonselective cation channels and causes further influx of Ca\(^{2+}\) as well as Na\(^{+}\), and efflux of K\(^{+}\) (Watkins and Olverman, 1987). The presence of excess EAAs causes the continual stimulation of these receptors, excess Ca\(^{2+}\) entry, and eventual Ca\(^{2+}\) overload (Choi, 1988). Heightened cytosolic Ca\(^{2+}\) causes deregulation of mitochondrial Ca\(^{2+}\) buffering and membrane permeability transition (Halestrap, 1999). Furthermore, mitochondria release reactive oxygen species and pro-apoptotic factors, leading to eventual neuronal death (Suh et al., 2007). A plethora of studies have attempted to block this cascade at different stages using various compounds, including NMDA antagonists (Sucher et al., 1997), poly-(ADP-ribose) polymerase 1 inhibitors (Suh et al., 2003), replacement of extracellular Na\(^{+}\) with cell impermeant cations (Rothman, 1985), and transient receptor potential channel blockade (Aarts et al., 2003).
Glucose deprivation results in selective neuronal death. Although Wyllie et al. (1980) proposed that all cell death may fit into either ‘apoptosis’ or ‘necrosis’, Olney (2003) argues that cellular death caused by excitotoxicity does not entirely conform to either category. Auer and colleagues (1985) observed that early lesions of neurons are marked by dendritic swelling, possibly due to water flux through open ion channels (Auer, 2004). Neurons are vulnerable to excitotoxicity, but glia and other non-neuronal cell types are spared due to a lack of excitatory receptors (Auer and Siesjö, 1988).

In 1984, Auer and colleagues documented the pattern of neuronal loss in hypoglycemic rats, concluding the areas most susceptible to hypoglycemia include the subiculum, caudate, CA1 hippocampus, crest of the dentate gyrus, and superficial layers of the cerebral cortex. In contrast, neurons in the brain stem, cerebellum, and spinal cord, seem to be resistant to hypoglycemic challenges. Auer speculated that excitotoxins may circulate through the CSF ventricles to produce the unique pattern of neurodegeneration.

1.3. Clinical Hypoglycemia

1.3.1. Introduction to Hypoglycemia

Hypoglycemia has been documented since the discovery of insulin in the early years of the 20th century (Banting et al., 1922; Paulesco, 1921). Although an uncommon event in the healthy population, hypoglycemia can be caused by hepatic/renal/cardiac failure (Fischer et al., 1986), hormonal deficiencies (Haymond et al., 1976), and insulinoma (Marks, 1992). It is, however, most prevalent in individuals who require glucose-lowering drugs, such as insulin and sulfonylurea, for treatment of diabetes mellitus (Cryer, 1997). Due to imperfect glucose-lowering
Drug regimens, combined with impaired glucose counter-regulation (discussed below), hypoglycemia is a common inadvertent (iatrogenic) consequence to diabetic therapy (Cryer, 1997). Even under ideal clinical conditions, 65% of patients with type 1 and 11% with type 2 diabetes suffered debilitating episodes of severe hypoglycemia during 6 years of intensive insulin therapy (Diabetes Control and Complications Trial [DCCT] Research Group, 1993). While the advent of new therapeutic strategies, including the use of continuous subcutaneous insulin infusion, short- and long-term insulin secretagogues, and behavioral education approaches, have improved patient glycemic control, hypoglycemia continues to be a feared and serious consequence to treatment. As Cryer et al. (2003) state, pending the cure of diabetes, elimination of iatrogenic hypoglycemia will require novel therapy that provides plasma glucose-regulated insulin replacement.

1.3.2. Defining Hypoglycemia

Creating definitive criteria for what constitutes an episode of hypoglycemia presents an immediate challenge for researchers. Biochemical definitions require an established glycemic threshold, below which is considered hypoglycemic. The American Diabetes Association (ADA) has suggested a blood glucose concentration of 3.9mM and below as representative of hypoglycemia (ADA Workgroup on Hypoglycemia, 2005), however this limit has been challenged as being too high (Strachan, 2007).

From a clinical perspective, biochemical definitions of hypoglycemia do not offer much value. Firstly, it is common in clinical practice to obtain blood samples from venous or capillary sources, which usually present lower blood glucose levels than arterial blood and can regularly
drop below 3.9mM after overnight fasts (Heller and Macdonald, 1996). Secondly, biochemical definitions do not account for symptoms associated with hypoglycemia. Attempts, however, to provide a comprehensive clinical definition are similarly met with frustration. The onset and type of symptoms seen during hypoglycemia is highly individualized (Cryer, 2008). Some patients can experience symptoms of hypoglycemia above 3.9mM while others, particularly those suffering from hypoglycemia unawareness, are symptom-free until blood glucose levels drop below 2mM (Boyle et al., 1988). Furthermore, distinguishing between “mild”, “moderate”, and “severe” hypoglycemia adds more complexity to the definition.

Perhaps the most commonly used method for hypoglycemia detection is based upon the early work of Allen Whipple. In 1938, Whipple outlined three criteria (known as Whipple’s triad) for the performance of pancreatic surgery on hyperinsulinemic patients (and are today used to diagnose hypoglycemia): symptoms are compatible with hypoglycemia, patient has a low plasma glucose concentration, and symptoms are relieved after plasma glucose has been raised.

In this thesis, a biochemical definition of hypoglycemia, as proposed by the ADA, was used. Hypoglycemia was defined as any blood glucose level below 3.9mM. Severe hypoglycemia was defined as any blood glucose level below 2mM.

1.3.3. Frequency and Impact of Hypoglycemia

The true frequency of hypoglycemia is difficult to estimate at least in part due to its varying definition, as previously discussed. Additionally, most episodes occur at home or work, without medical staff involvement, and with poor subsequent recall from patients (Strachan, 2007).
Finally, quality of glycemic control and the presence of hypoglycemia unawareness in individual patients add further influences to the frequency of hypoglycemia experienced.

A study by Pramming and colleagues (1991), using the onset of hypoglycemic-associated symptoms as a marker, discovered a frequency averaged to about 94 episodes per patient per year (patient-year) for type 1 diabetics. Janssen et al. (2000) used a biochemical definition (hypoglycemia as having below 3.5mM blood glucose) and reported a frequency of 160 episodes per patient-year. Cryer (2006) has estimated a typical type 1 diabetic patient will experience two episodes of symptomatic hypoglycemia each week. According to the United Kingdom Prospective Diabetes Study (UKPDS, 1998) Group, type 2 diabetics had a lower frequency of hypoglycemia than type 1, however episodes became more frequent and limiting to euglycemic control with increasing time after diagnosis.

In as many as 40% of cases, it is not possible to identify the exact cause for an episode of hypoglycemia (Potter et al., 1982), however certain risk factors have been associated with increased risk for hypoglycemia. The DCCT (1993) has provided substantial evidence that intensive insulin therapy can cause an increase (in this case, a three-fold increase) in the vulnerability to hypoglycemia. A study by Davis et al. (1997) determined that longer duration of diabetes and being of young age were also risk factors to developing severe hypoglycemia.

Hypoglycemia can pose a great impact upon both physical and psychological wellbeing. At the very least, hypoglycemia can be a nuisance, but it may also lead to a fear of recurrent episodes, feelings of guilt resulting from aforementioned fear, social ostracism, high levels of anxiety, and
depression, (Cryer, 2006) in addition to potentially being a significant economic strain (Gonder-Frederick, 1997).

1.3.4. Glucose Counter-regulation and Hypoglycemia-associated Autonomic Failure

Within healthy individuals, there is a multi-tiered defense against aberrant changes in blood glucose concentrations. The sequential glycemic thresholds for these responses have been previously characterized (Mitrakou et al., 1991; Schwartz et al., 1987). The first response is a decrease in insulin secretion once blood glucose levels drop below 4mM. Increases in glucagon and epinephrine occur next if lowered insulin levels do not rectify blood glucose levels. With prolonged hypoglycemia, activation of the autonomic nervous system further increases epinephrine secretion and creates characteristic warning signs and symptoms (described below). As blood glucose levels drop below 2mM, cerebral blood flow increases to enhance glucose delivery to the brain (Thomas et al., 1997). Finally, Tappy and colleagues (1999) show that with severe and prolonged hypoglycemia, the liver is able to increase glucose production in the absence of detectable hormonal stimulation (autoregulation).

The warning signs and symptoms of hypoglycemia can be categorized into neurogenic and neuroglycopenic responses. Neurogenic responses are largely caused by sympathoadrenal activation (DeRosa and Cryer, 2004). Adrenergic surge causes palpitations, tremor and heightened anxiety (“fight or flight” response) while cholinergic activation can trigger sweating, increased hunger, and paresthesia. Neuroglycopenic symptoms are the direct result of reduced glucose utilization by neurons, and can cause cognitive impairments, behavioral changes, and
psychomotor abnormalities (DeRosa and Cryer, 2004). Despite these broad characterizations, symptoms of hypoglycemia are not universal, and so diabetic patients typically learn their own unique responses to hypoglycemia through experience (McAulay et al., 2001). If treatment is not sought, however, severe hypoglycemia can ensue and bring about coma and/or seizure manifestations.

The intricate defense system against low glucose concentrations ensures that clinical hypoglycemia remains a rare occurrence in the general population. In fully developed type 1 diabetes, however, defects in this system result in hypoglycemia-associated autonomic failure (HAAF). Chronically elevated blood glucose levels desensitize glucose sensing cells, which in turn reduce autonomic responses to blood glucose fluctuations and increase likelihood of hypoglycemia unawareness (Bolli et al., 1984; Dagogo-Jack et al., 1993; Fig. 2). Furthermore, in 1973, Gerich and colleagues observed that plasma glucagon levels did not rise in type 1 diabetic patients even with the presence of circulating insulin, while levels did rise in non-diabetic control subjects. This suggested that exogenous insulin treatment may not stimulate α cell glucagon secretion (Gerich et al., 1973).

In essence, HAAF increases the chance of recurrent hypoglycemia, which in turn exacerbates HAAF, continuing a vicious cycle of defective glucose counter-regulation. Indeed, a study has revealed that even one episode of hypoglycemia can impair glucose counter-regulation (Shum et al., 2001), although strict avoidance of hypoglycemia for 2-3 weeks can attenuate such defects (Fanelli et al., 1994).
Figure 2. Body defenses against hypoglycemia. ACh, acetylcholine, NE, norepinephrine, PNS, parasympathetic nervous system; SNS, sympathetic nervous system. (Cryer PE. 2008. The barrier of hypoglycemia in diabetes. Diabetes 57(12): 3169-76. © by American Diabetes Association. Adaptation by permission of copyright holder).
1.3.5. Hypoglycemia in Children

Hypoglycemia is seen in diabetic individuals of all ages, but studies have consistently shown that prepubescent children and infants are most susceptible to its development (Danne et al., 1994; Davis et al., 1997; DCCT, 1993; Hershey et al., 2005 Schultz et al., 1999).

The cause of this greater vulnerability remains unclear, but some factors have been suggested. Kennedy and Sokoloff (1957) observed that cerebral blood flow in young children (3-11 years old) was 106mL/100g·min and O₂ consumption was 5.2mL/100g·min (approximately 50% of total body O₂ consumption), both of which were significantly higher than observed in adults (62 and 3.5mL/100g·min, respectively). The high cerebral metabolic rate suggests changes in glucose delivery to the brain may cause deficits either quicker or more severely in children than in adults.

Furthermore, lifestyle differences may also play a role in hypoglycemia vulnerability. Davis (1997) suggested that a child’s irregular eating habits, sporadic physically active nature, and potential inability to identify signs of hypoglycemic onset, can lead to higher frequencies of hypoglycemia.

Finally, an abstract has detailed preliminary evidence that onset of HAAF may occur quicker in children than in adults (Caplin et al., 2000), exacerbating the risk of severe hypoglycemia.
1.3.6.  Hypoglycemic Seizures

The most feared, and perhaps most common (Kaufmann, 1998; Pocecco and Rofani, 1998), symptom of severe hypoglycemia is seizure generation.

Seizures, as defined by the International League Against Epilepsy, is the “transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” (Fisher et al., 2005). They have various etiologies and efforts to categorize them date back to classic medical literature (Wolf, 1985). Seizures can range from being mild absence seizures to the uncontrolled movements of generalized tonic-clonic seizures. The electroencephalogram (EEG) is the most used and informative laboratory test for the diagnosis of seizures. It is used to characterize the abnormal brain electrical activity, identify the type of seizure, and locate the area of seizure focus (Goldensohn, 1996).

Seizures induced by hypoglycemia have been described as showing ictal spike and wave activity and are believed to be generalized in origin (Velísek et al., 2008; Pitkänen et al., 2005). The lack of detailed electroencephalographic or behavioral descriptions of hypoglycemic seizures underscores the paucity of published studies focusing specifically upon this phenomenon.
1.4. Select Studies on Hypoglycemia

1.4.1. *In vitro* Studies

1.4.1.1. Hypoglycemic Seizures

A few groups have attempted to study hypoglycemic seizures *in vitro*. Using an intact hippocampal preparation, Abdelmalik and colleagues (2007) sought to determine the effects of hypoglycemic-induced epileptiform activity in immature mice. It was discovered that lowering glucose from 15mM to 4mM evoked spontaneous seizure-like activity in the hippocampus, and such activity always preceded a ~90% reduction in evoked synaptic field potentials. The seizure-like activity was prevented by both NMDA (MK801, APV) and non-NMDA (CNQX) antagonists, in addition to the anticonvulsant midazolam (a benzodiazepine derivative).

In contrast to Abdelmalik, Kirchner et al. (2006), using 400µm rat entorhinal cortex-hippocampus slices, was unable to elicit epileptiform discharges in varying low glucose environments (5, 2, and 1mM), despite showing involvement of the hippocampus in hypoglycemic seizure generation *in vivo*. Other studies have demonstrated the hippocampus is prone to seizure generation and sensitive to seizure-induced damage (Abdelmalik et al., 2005; Haut et al., 2004). These findings suggest that although the exact role of the hippocampus within the pathophysiology of hypoglycemic seizures remains to be elucidated, it is likely this structure has an important function.
1.4.1.2. Effects on Long-term Potentiation

Cognitive dysfunction associated with hypoglycemia has led to the *in vitro* examination of long-term potentiation (LTP) during low-glucose perfusion. Sadgrove and colleagues (2007) reported that LTP induction and maintenance from the hippocampal CA1 region declined in a glucose concentration-dependent manner. Izumi and Zorumski (1997) found that in moderate to mild (2-3.3mM) levels of hypoglycemia, tetanus-induced LTP could be preserved through NMDA receptor or nitric oxide synthase inhibition, yet these measures were ineffective during severe (<2mM) hypoglycemia. Another study demonstrated that repeated hypoglycemic (2mM) episodes in rats inhibited the induction of LTP, despite showing normal baseline evoked synaptic responses (Yamada et al., 2004).

1.4.1.3. Changes in Ca$^{2+}$ concentrations

Since Ca$^{2+}$ has been implicated in hypoglycemia-induced neurodegeneration, studies from the Heinemann laboratory have investigated intra- and extracellular concentrations of Ca$^{2+}$ in the presence of low-glucose ACSF. In rat hippocampal slices, stimulation of Schaffer collaterals under low-glucose conditions resulted in an augmentation of presynaptic Ca$^{2+}$ entry (Alici and Heinemann, 1995), which may initiate excitotoxicity. In another study, extracellular Ca$^{2+}$ levels were compared in the CA1 region of adult and juvenile (20-22 days old) rat slices. Low glucose-induced declines in extracellular Ca$^{2+}$ concentrations were delayed in juvenile animals, suggesting that neurons show more resistance to neurodegeneration in early postnatal development (Alici et al., 1998).
1.4.2. *In vivo* studies

1.4.2.1. Hypoglycemic Seizures

Velísek et al. (2008) examined hypoglycemic seizures using a rat model. They demonstrated that overnight fasting predisposes rats to insulin-induced hypoglycemic seizures. Using labeled 2-deoxyglucose uptake studies, they observed increased metabolic activity in the substantia nigra pars reticulata preceding hypoglycemic seizures. Thus, this structure may play a role in hypoglycemic seizure regulation.

Del Campo and colleagues (2009) attempted to correlate EEG activity in the hippocampus and frontal neocortex with seizure-like behavior in a hypoglycemic rat model. They observed behavioral seizures in some (8 of 12) animals, but did not correlate well with EEG activity. They, hence, suggest that seizure-like behaviors may originate from deeper brain structures.

1.4.2.2. Effect on Cognition

Studies examining hypoglycemia and its potential detrimental effect upon learning and memory remain inconclusive about the results. The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC, 2007) examined 1441 diabetic patients in an 18-year study on cognitive decline associated with diabetes. The results showed no evidence of significant long-term cognitive decline despite relatively high rates of recurrent severe hypoglycemia and accompanying coma and seizures. Although the findings of this study are well substantiated, few young children or adolescents were included in
the trial, hence the possibility that impairments occur within this subgroup can not be ruled out (Cryer, 2008).

Studies focusing specifically on children find more convincing evidence of cognitive decline associated with hypoglycemia. Northam (1998, 2001) has shown that neuropsychological profiles of diabetic children with a history of hypoglycemia show decline in processing speed, acquisition of new knowledge, and conceptual reasoning skills, consistent with impairments in temporal brain regions. A long-term prospective study by Schoenle and colleagues (2002) observed a significant decline in verbal intelligence in diabetic boys between 7 and 16 years of age. Finally, Rovet et al. (1999) echoed previous results by evaluating type 1 diabetic children with hypoglycemia 7 years post-diagnosis, and showed they had deficits on perceptual, motor, memory and attention tasks.

*In vivo* animal models have also been used to study cognitive effects of hypoglycemia. McNay and colleagues (2006) used a rodent model to evaluate the effects of weekly episodes of hypoglycemia on cognitive tasks over a 12 month period. Interestingly, recurrent hypoglycemia appeared to slightly improve cognitive performance while at euglycemia, but impaired functions in hypoglycemic conditions. Furthermore, upon examination *in vitro*, McNay observed improved GABAergic signaling in the hippocampus in normoglycemic conditions from recurrent hypoglycemic animals as compared with controls, but significant loss of GABAergic signaling in low glucose environments.
1.4.2.3. EEG in humans

To understand the principles of the EEG, consider a simplified version by recording the electrical properties of a single neuron (Fig. 3). The depolarization of a localized section of neuronal membrane creates an inward rush of positive ions (inward current), creating a current ‘sink’. These ions then diffuse to distal sections of the neuronal membrane and are actively transported back into the extracellular space. The outward flow of positive ions (outward current) creates what is known as a current ‘source’. The pairing of a current sink and source creates a current loop that serves as the fundamental basis for recording electrical activity in the brain. If an extracellular microelectrode is placed near the site of the current ‘sink’, a negative deflection will be recorded since positive current is moving away from the electrode. Alternately, if the microelectrode is placed near a current source, a positive deflection is recorded (Fig. 3). The EEG signal is, therefore, the weighted average of the extracellular current produced by the numerous neural circuits within the brain (Kandel, 2000). Those circuits closer to the recording electrode will contribute more significantly to the EEG signal.
Typical non-invasive scalp EEG activity in humans oscillates at multiple frequencies, associated with various synchronized neural networks in the cerebral cortex and other structures. Perhaps the most prominent oscillations in humans are α waves, which are fairly regular and have a frequency between 8-13Hz (Kandel et al., 2000). It typically occurs over the occipital region when the person is awake but in a relaxed state. A more irregular, yet still prominent rhythm is known as β waves, which are low-amplitude and have a frequency between 13-30Hz. They are usually seen in awake and alert adults as well as during rapid-eye movement sleep. The θ and δ waves are seen between 4-7Hz and 0.5-4Hz, respectively. Both rhythms occur during sleep and it is believed the θ wave may be involved with learning and memory (Buzsáki, 2006; Kandel et al. 2000).

In humans, studies have shown that hypoglycemia brings about a decrease in α wave activity while increasing slow δ waves (Pramming et al., 1988; Tallroth et al., 1990). It was suggested that increased amplitude and decreased frequency of rhythmic bands correlates to the sympathoadrenal response to hypoglycemia (Auer, 2004). Severe hypoglycemia and increased δ waves may be the result of a protective, energy-conserving decrease in overall neuronal activity (Auer et al., 2004). Other studies have shown that in individuals without diabetes, altered latencies of evoked cortical field potentials were associated with insulin-induced hypoglycemia (Blackman et al., 1990; Pozzessere et al., 1991). Soltezs et al. reported in 1989 that in diabetic children, who are most susceptible age group (0-12 years) to hypoglycemic seizures (Engel et al., 2007), there is a nearly four-fold increase in EEG abnormalities in relation to controls. Diabetic children had consistent decreases in α waves and increases in non-rhythmic and paroxysmal activity. Greater deviations from normal EEG tended to be accompanied by behavioral disturbances and a history of severe hypoglycemia (Eeg-Olofsson et al., 1977).
1.4.2.4. EEG in animals

In animal models, evolution of EEG activity tends to parallel what is seen in humans. Suh et al. (2003) observed that within the first 30 minutes of hypoglycemia in rats, subdural EEG showed rhythmic patterns decreased in frequency but increased in amplitude. As hypoglycemia continued, paroxysmal activity became present, then burst-firing and suppression patterns until the EEG became isoelectric (with blood glucose levels below 0.5mM). It has interestingly been noted for decades that aberrant changes in EEG activity occur without any concurrent drops in brain ATP or phosphocreatine levels (Norberg and Siesjö, 1976). Only at the onset of EEG isoelectricity is there a significant decreased seen in ATP and phosphocreatine levels. Sustained ATP and phosphocreatine reserves may be due to energy-conserving suppression of neuronal signaling and glycogenolysis of astrocytic glycogen reserves.

Very few studies have examined the EEG profile of hypoglycemic seizures. Kirchner et al. (2006) examined the effects of hypoglycemia upon adult rats subjected to the pro-convulsant flurothyl. Moderate hypoglycemia appeared to exacerbate behavioral convulsions associated with flurothyl. Additionally, intrahippocampal recording electrodes were used to record seizure activity during insulin-induced hypoglycemia. It was demonstrated that severe hypoglycemia per se was frequently associated with hippocampal activation. The study, however, did not investigate whether repeated episodes of hypoglycemia may increase hippocampal activation or increase the frequency of its association with hypoglycemic seizures. Furthermore, juvenile rats were not considered in this work.
A more recent study examining hypoglycemic seizures was published by Velísek et al. (2008). Adult rats were subjected to insulin-induced hypoglycemic seizures and intracranial electrodes were placed in the sensorimotor cortex, dorsal hippocampus, anterior substantia nigra and pedunculopontine tegmental nucleus. Between overnight-fasted and nonfasted rats, a significant increase in barrel rotations was observed in the fasting group during insulin-induced hypoglycemia. EEG activation occurred nearly simultaneously across all regions recorded during hypoglycemic seizures. Imaging data obtained through 2-deoxyglucose uptake and c-Fos expression indicate that the substantia nigra, superior colliculus and pedunculopontine tegmental nucleus play important roles in controlling seizure-prone neural networks. A detailed examination of the EEG profile, including alterations in prominent rhythmic activities, was not reported in this study. Furthermore, as with other studies, only adult animals (youngest were 55 days old) were considered in these experiments.

1.5. Rationale

1.5.1. EEG

Little is known concerning the pathophysiology of hypoglycemic-associated abnormalities, especially seizures. Most studies describe such seizures as generalized (Kirchner et al., 2006; Suh et al., 2003; Velíšková et al, 2007), but its site of origin, mechanism of propagation, and structures involved remain to be elucidated. Furthermore, correlation of behavioral and electrographic abnormalities has yet to be accomplished in young animals. Detailed examination of EEG may help elucidate electroencephalographic patterns associated with mild and severe symptoms of hypoglycemia. Also, a comparison between non-diabetic and diabetic juvenile animals may yield insights into the impaired response of the diabetic brain to hypoglycemia.
1.5.2. Hippocampus and parietal cortex

An early study reported that hypoglycemic seizures may originate from the hippocampus (Tokizane and Sawyer, 1957). *In vitro* work has also shown the intact hippocampus to generate ictal activity in low-glucose environments (Abdelmalik et al., 2007). Furthermore, cognitive impairments associated with hypoglycemia (INTRODUCTION 1.4.2.2.) also suggest the hippocampus is affected by hypoglycemia. Finally, neurodegeneration attributed to hypoglycemia also appears to affect parts of the hippocampus first (Auer et al., 1984). These studies suggest an important role for the hippocampus in the hypoglycemia-induced brain function abnormalities, hence warrant EEG investigation.

As Auer et al. (1984) observed, the cortex is vulnerable to hypoglycemic-induced damage. Furthermore, a study has suggested hypoglycemic seizures are associated with exacerbated brain dysfunction (Abdelmalik et al., 2007). Hence, an investigation of cortical regions during repeated hypoglycemia was conducted.

1.5.3. Juvenile animals

Although *in vivo* and *in vitro* animal models have been established to simulate hyperinsulinemic hypoglycemia, there have been virtually no studies that have examined aspects of hypoglycemia or hypoglycemic seizures within a juvenile animal population. The use of immature animals, as oppose to adult animals, correlates more strongly with young diabetic children, who are most susceptible to the detrimental consequences of hypoglycemia, as previously discussed (INTRODUCTION 1.3.5.).
1.5.4. Repeated hypoglycemia

A previous study which examined EEG in adult rats during hypoglycemic seizures found poor correlation between behavioral and EEG events (Del Campo et al., 2009), yet this study only examined a single episode of hypoglycemia per animal. Previous studies have demonstrated that repeated bouts of hypoglycemia can alter cognitive performances (McNay and Sherwin, 2004; McNay et al., 2006), as well as disrupt glucose regulatory systems (INTRODUCTION 1.3.4.). HAAF increases the likelihood of severe hypoglycemia, which may in turn increase seizure susceptibility and other hypoglycemic-associated abnormalities. In addition, investigating repeated hypoglycemic events in animal models correlates with the clinical condition, where diabetic patients experience about two symptomatic hypoglycemic episodes per week (Cryer, 2008).
Chapter 2
Objectives and Hypotheses

2.1. Main Objective and Hypothesis

The main objective of this thesis is to: firstly, describe and compare the progression of hippocampal and cortical EEG, using intracranial electrodes, during repeated hypoglycemic episodes in juvenile diabetic and non-diabetic mice. Secondly, to investigate the severity of behavioral seizures induced with repeated hypoglycemic episodes and note any differences seen between juvenile diabetic and non-diabetic mice.

The overall hypothesis is that repeated hypoglycemic episodes will exacerbate any EEG abnormalities seen in the hippocampal and cortical EEG as well as increase the severity of behavioral seizures observed. Furthermore, these abnormalities will be more prominent in the juvenile diabetic animal group than the non-diabetic group.

2.2. Specific Hypotheses

More specifically, the experiments of this thesis will aim to test the following hypotheses:

1. Insulin-induced hypoglycemia will trigger behavioral seizures more frequently in juvenile mice in comparison with adult mice. This will correlate with clinical findings that consistently report young diabetic children as more prone to seizure development than adults (Engel et al., 2007; Rovet and Alvarez, 1997).

2. During recurrent hypoglycemia, each hypoglycemic episode will increase the severity of hypoglycemic behavioral seizures. This hypothesis is based upon previous observations
that repeated hypoglycemia impairs glucose counter-regulation and hence increases the possibility of severe hypoglycemia (Cryer PE, 2008). Similarly, we predict diabetic animals, which are known to have aberrant glucose counter-regulation, will show further exacerbated behavioral seizures compared with non-diabetic animals.

3. Repeated hypoglycemic episodes will worsen hypoglycemic-induced reductions in blood glucose, possibly due to impaired glucose counter-regulation.

4. During hypoglycemia, the hippocampal θ rhythm will transiently disappear. Alterations (amplitude or frequency) in the θ rhythm will be present after repeated hypoglycemia. This hypothesis is based upon the finding that hypoglycemia affects cognitive functions (Davis et. al, 1998; Rovet et. al, 1993) and the hippocampal θ wave is associated with exploratory behavior (Buzsáki, 2006).

5. Behavioral seizures will correlate with ictal discharges in the hippocampal and cortical EEG electrodes. Repeated hypoglycemia will exacerbate these ictal discharges by increasing their frequency and duration. Diabetic animals will show greater occurrences of hypoglycemic seizures than non-diabetic animals. This hypothesis is based upon previous in vitro data demonstrating the seizure generating ability of the hippocampus (Abdelmalik et al., 2007).
Chapter 3
Methods

3.1. Animals

C57 black/6N-strain male mice were obtained from Charles River Breeding Farms (Montreal, Quebec, Canada) between postnatal day 18-21. They were housed in a vivarium with a 12-hour day/night cycle (lights on at 6am to 6pm) and at a constant temperature of 23±1°C. Rodent chow and water were available *ad libitum*. All experimental procedures were approved by the local animal care committee and adhered to the guidelines set by the Canadian Council on Animal Care.

3.2. Drugs

Avertin was prepared by dissolving 2.5g of 2,2,2-tribromoethanol with 5mL of tert-amyl alcohol, then diluted to 2.5% w/v in distilled water. The solution was stored in a 4°C refrigerator. Buprenorphine (Buprenex) was used during animal implantation and streptozotocin (STZ; 2-deoxy-2[3-methyl-3-nitrosoureido]-D-glycopyranose) was used to induce diabetes. Aforementioned drugs and chemical compounds were purchased from Sigma Aldrich (Oakville, ON, Canada).

Induction of hypoglycemia was done using either normal (Humulin R, Eli Lily, Toronto, ON, Canada) or fast-acting insulin (Humalog, Eli Lily). Insulin was obtained from the clinical pharmacy at the Toronto Western Hospital.
3.3. In vivo Experiments

The methods described in METHODS (3.1.1.) and (3.3.2.) have been published in the Journal of Neuroscience Methods (Wu et al., 2008). The author of this thesis is a co-author on the publication and has experience in the construction and implantation of the electrode assembly.

3.3.1. Electrode Construction

Each intra-cranial electrode was assembled using a recording wire and connecting pin. The recording wire was made from polyimide-insulated stainless steel wire (outer diameter 0.25mm and inner diameter 0.21mm, Plastics One, Roanoka, VA, USA, initial length of 15mm). When measured from a piece of 20mm in length, the resistance of the stainless wire was 0.68±0.03Ω. The wire was scratched at one end to remove the insulating layer for soldering. A connecting pin (6mm in length) was cut from a 21-gauge stainless steel tube (Plastics One). A small cut was made at one end of the pin for better contact between the pin and epoxy to be applied. The recording wire was put in the connecting pin and soldered together with the pin. A soldering liquid (Soldering Liquid Flux, Certanium Alloys and Research Company, Cleveland, OH, USA) was used to achieve a better contact between the stainless recording wire and connecting pin. A plastic base of 5x5mm was cut from the curved portion of a plastic weighing dish (VWR International, Mississauga, ON, Canada). Because we used three electrodes in our experiments, three small holes (0.3mm diameter) were made in the plastic base and their positions matched to the stereotaxic coordinates of desired recording sites. Three recording electrodes were put through the base, and then fastened in place using epoxy (5-min epoxy, Lepage, Henkel, Brampton, ON, Canada). Epoxy was laid on the top side of the plastic base while the bottom
side was clean, ensuring good contact with skull during electrode implantation. After the epoxy hardened, the recording wires were cut to match the depths of recording sites. The assembled electrodes were cleaned with 75% alcohol and stored in a sterilized glass bottled prior to implantation. The total weight of the electrode assembly was ~90mg.

3.3.2. Surgery and Electrode Implantation

All surgical instruments were sterilized prior to use and all injections were made using a 30-gauge needle. Animals were anesthetized with Avertin (intraperitoneal [i,p.], 2.5%, 0.2mL/10g). Previous studies show Avertin being effective at inducing surgical anesthesia in small laboratory animals, despite some reservations of post-operative fatalities (Norris and Turner, 1983; Green CJ, 1975). In these experiments, Avertin reliably induced surgical level anesthesia in mice and were associated not associated with any animal deaths. Once surgical anesthesia was reached, animals were placed onto a stereotaxic frame and held by a mouse adaptor (Kopf Instruments, CA, USA). The animal’s head was swabbed with antiseptic betadine solution, then the skin atop the head was incised and three small holes (0.5mm diameter) were drilled in the skull at specific stereotactic coordinates (see below). The underlying dura was gently opened using a fine needle. The electrode assembly was then lowered atop the skull. The glue has a cure time of several seconds and high bond strength. The sites of EEG recording were the hippocampal CA1 area (Bregma -2.3mm, lateral 1.7mm and depth 2.0mm) and contralateral parietal cortex (Bregma -0.8m, lateral 1.8mm and depth 1.5mm). A reference electrode was placed near the cortical electrode (Bregma -3.8mm, lateral 1.8mm, depth 1.5mm; Franklin and Paxinos, 1997). A cyanoacrylate-based glue (Insta-cure+, BSI Adhesives, CA, USA) was used to fasten the electrode assembly atop the skull as well as secure the incised skin such that skull exposure to
the external environment was minimized. The animal was allowed to recover for at least 3 days before further experimentations, during which time no behavioral abnormalities were noticed.

Figure 4. Schematic of electrode assembly and implanted mouse. A, plastic base with 3 holes matched to desired recording and reference sites. Illustration (B) and image (C) of completed electrode assembly. D, image of implanted juvenile mouse. (portions of this Figure from Wu C, Wais M, Sheppy E, del Campo M, Zhang L. 2008. A glue-based, screw-free method for implantation of intra-cranial electrodes in young mice. J Neurosci Methods. 171(1): 126-31. © by Elsevier Inc. Adaptation by permission of copyright holder).
3.3.3. **Insulin-induced hypoglycemia**

Between postnatal day 24-27 (3-6 days after surgery), implanted animals were fasted for a 2-hour period. This interval was selected so as to normalize blood glucose levels in case some animals had recently eaten.

After the fasting interval, animals were administered fast-acting insulin (*i.p.*, 20UI/kg). The animals were monitored for 90 minutes following insulin injection, after which time they received glucose (*i.p.*, 1g/kg, dissolved in saline). EEG was continuously recorded from 30 minutes before insulin injection to 30 minutes after glucose rescue. Age-matched implanted control mice received equi-volume of saline (100uL, *i.p.*), and their EEG was similarly recorded. Insulin injections were repeated 7 and 14 days after the first injection in an attempt to examine the effects of recurrent hypoglycemic episodes. Repeating insulin-induced hypoglycemia on a weekly basis in a rodent model has previously been suggested to correlate with the clinical setting (McNay et al., 2006). Blood glucose was measured at different time points throughout the recordings to ensure achievement of hypoglycemia and restoration of euglycemia.

Induction of hypoglycemia in non-implanted mice was performed in a similar manner, except that regular insulin (Humulin-R) was used and varying doses were administered (rationale for switching between fast-acting and regular insulin provided in **RESULTS 4.1, 4.3.**)

3.3.4. **EEG recordings**

EEG was recorded using two extracellular amplifiers with extended head-stages (Model-300, AM Systems Inc., WA, USA). The head stages were secured about 10cm above the animal
housing cage and connected to the electrode assembly using soft wires and connecting pins (gold wire contacts, Fine Scientific Tools Inc., Vancouver, Canada). The soft wire was isolated from a multi-wire cable (CW6300, Cooner Wire, CA, USA).

Signals were recorded in a frequency band of 0.05-1,000Hz and amplified 1000x before digitization (digitization rate of 60kHz; Digidata 1300, Molecular Devices, CA, USA). Data acquisition and storage were done with pCLAMP software (Molecular Devices) and analyzed offline. For spectral analyses, original data were treated with a band-pass filter (Bessel) of 1-500Hz. The main peak of the spectral plot was considered as the dominant frequency of EEG rhythmic activity. For amplitude measurement, EEG data were treated with a band-pass filter (Bessel) of 0.5-500Hz. EEG signals from a 2-minute recording period were automatically detected with a voltage threshold of 0.01mV and temporal resolution of 0.1msec and were used to obtain mean amplitudes.

### 3.3.5. Behavioral Assessment

A video camera (Canon, model ES400V) was used to continuously monitor animals’ behavior before and after insulin injection. Data were stored on videocassette tapes and analyzed offline. The severity of behavioral (motor) seizures was determined using a scale for primary generalized seizures (Velíšková, 2006). This scale was used because previous reports generally agree that hypoglycemia-induced seizures are generalized (Velisek et al., 2006; 2008).

The scale measures severity based upon a 0 to 5 ranking. Briefly, stage 0 or 0.5 refers to no abnormality or minor abnormalities such as excessive sniffing or grooming. Stage 1 or 2 refers to isolated myoclonic jerks or atypical clonic seizing. Stage 3 represents fully developed
bilateral forelimb clonus, including tonic components and body twists. Stage 4 or 5 corresponds to tonic-clonic seizing with suppressed tonic phase or fully developed tonic-clonic convulsions.

3.3.6. Blood Glucose Measurements

Blood collection was adapted from the ‘Tail Snip’ protocol found in the Jackson Laboratories Surgical Operating Procedures. Mixed arterio-venous blood was collected from animals by cutting the tip of the tail (≤1mm) using a scalpel blade. Silver nitrate was administered to lesion site after blood collection to aid in sealing wound. Samples obtained were 10±5µL. In successive blood collections, a scalpel blade was used to scrape off scab and the tail was gently massaged to withdraw blood. Blood samples were collected prior to, and at 20, 40, 60, 80 minutes post-insulin injection, or 30 minutes after an intraperitoneal injection (1g/kg) of glucose-containing solution. Blood glucose was measured with a laboratory glucose analyzer with a measuring range of 0-22.2mM (HemoCue Glucose 201 Analyzer, HemoCue Inc., CA, USA).

3.3.7. Diabetes Induction

Animals were made diabetic using a low-dose streptozotocin (STZ) administration protocol (Ichinose et al., 2005). Animals were given three intraperitoneal injections of 100mg/kg STZ (dissolved in 10mM sodium citrate) over the course of 48hrs. Animal weight and peripheral blood glucose was recorded 2-, 4-, 6-, and 8-days proceeding STZ administration. Control animals received equi-volume i.p. injections of citrate buffer only.
3.4. Statistics

Data analysis was done using pCLAMP and SigmaStat software (Systate Software Inc., San Jose, California, USA, version 3.0). Unless otherwise noted, data are presented as mean values ± the standard error of the mean (SE).

The Fisher’s exact test was used to determine significance between seizure probabilities in juvenile and adult mice. For seizure severity data, the Friedman test was conducted with the Holm-Sidak method for multiple comparisons. EEG amplitude and frequency data were treated with the Student’s t-test (paired and unpaired) and analysis of variance (ANOVA) where indicated. A p value less than 0.05 was deemed significant in all statistical tests.
4.1. Hypoglycemia-induced behavioral seizures occur more frequently in juvenile mice than adults

It was hypothesized that insulin-induced hypoglycemia would trigger hypoglycemic seizures more frequently in juvenile mice as compared with adults. Non-implanted juvenile (21-28 days old) and adult (10 months old) mice were administered regular insulin, with a half-life of 3 hours, and monitored for 90 minutes post-injection. Seizures were defined as any convulsive activity with a severity score of 1 or more. At a dose of 15UI/kg, the juvenile mice had a high probability of displaying insulin-induced behavioral seizures, with 16 of 21 mice seizing. This was in contrast to adult mice, where only 2 of 10 animals underwent convulsions (p<0.01, Fisher’s exact test; Fig. 5). Further investigation revealed that the frequency of behavioral seizures in juvenile animals was dose dependent: at 5UI/kg, 4 of 12 mice exhibited hypoglycemic seizures. This ratio was increased at 10UI/kg, where 12 of 16 mice showed convulsive behaviors. The dosage of insulin used in mice is similar to those used in other studies (Anuradha et al., 2004). A limitation to this experiment, however, was that blood glucose levels were not examined between adult and juvenile animals. Although weight-adjusted equivalent doses of insulin were administered to both sets of animals, there could be potential differences in glucose handling between developing and mature mice which may contribute to the differences seen in behavioral seizure susceptibility.

These results are consistent with the stated hypothesis, and provide a reliable juvenile model of hypoglycemia to perform additional experiments. Previous work in vitro has shown increased
susceptibility in young mice to hypoglycemic seizures (Abdelmalik et al., 2007). Clinical studies repeatedly show young children as most likely to develop adverse effects to hypoglycemia (Davis et al., 1997; DCCT, 2004; Northam et al., 1998). The reason behind heightened seizure susceptibility in the young remains open to discussion, with some suggesting that, due to higher brain glycogen content at young age in animals (Buckner and Biesold, 1981), young animals are able to sustain the high-energy requirements of seizures (Dalsgaard et al., 2007) in the face of decreased systemic glucose supply.

**Figure 5.** Percentage of animals showing convulsive behaviors (defined as having a score above 1 on the scale for primary generalized seizures, Velíšková et al., 2006) when subjected to hypoglycemia with 5, 10, 15UI/kg insulin. At 15UI/kg, juvenile (21 day old) mice were compared with adult (10 month old) mice. *, p<0.01, Fisher’s exact test.
4.2. STZ-treated juvenile mice develop chronic hyperglycemia within 8 days

STZ is a broad-spectrum antibiotic which causes the selective destruction of pancreatic β cells (Rossini et al., 1977). It was administered, i.p., to juvenile (16 day old) mice to simulate type 1 diabetes. The protocol, adopted from Ichinose et al. (2005), called for mice to receive 3 injections of 100mg/kg STZ within a 48-hour interval. STZ was dissolved in 10mM sodium citrate buffer. On day four, basal blood glucose levels became significantly higher in STZ-treated animals than controls (9.4±0.4mM vs. 7.9±0.7mM; p<0.05). By day 8, all STZ-treated animals had blood glucose levels ≥15mM, and were thus considered diabetic (Chari et al., 2008). The average blood glucose level was 16.7±0.5mM, significantly higher than control animals (p<0.001, Fig. 6). The weight of STZ-treated animals increased from 13.1±0.4g to 17.7±0.5g over the course of the STZ protocol, which was not significantly different from weight gained by control animals. Weight gain is expected as the animals are juvenile.

It is of clinical relevance to use diabetic animal models, as oppose to healthy animals, when investigating hypoglycemia and hypoglycemic seizures. Hypoglycemia can affect EEG and cognitive function in both healthy and diabetic individuals (Tallroth et al., 1990; McCrimmon and Frier, 1994), but hypoglycemia is rare in healthy individuals while being prominent in the diabetic community (Cryer, 1997).
4.3. Repeated insulin-induced hypoglycemia exacerbates behavioral seizure severity in non-diabetic and diabetic juvenile mice

In experiments involving non-implanted mice (RESULTS 4.1.) 15UI/kg of regular insulin was used to achieve hypoglycemia. Although implanted animals did not show any behavioral abnormalities during basal conditions, the use of 15UI/kg of regular insulin surprisingly did not produce behavioral seizures within the 90 minute episode of hypoglycemia. Repeated episodes of hypoglycemia, similarly, did not produce behavioral seizures at the frequency noted in
**RESULTS 4.1.** Although investigating the reason behind this discrepancy is beyond the scope of this thesis, this issue is addressed in **DISCUSSION 5.4.** In a second set of experiments, fast-acting insulin (Humalog, half-life of 90 minutes) at a dosage of 20UI/kg (which is within the range used in other animal models [Del Campo et al., 2009; Linardi et al., 2000; Velísek et al., 2008]) produced more consistent behavioral seizures and was used for the remainder of the experiments.

Each insulin-induced hypoglycemic episode was separated by 7 days. Animals fully recovered after each episode of hypoglycemia without any observations of spontaneous convulsions or atypical behavior. One STZ-treated animal, however, died within 24 hours of the second insulin administration, possibly due to an error in glucose rescue injection.

Behavior abnormalities in both diabetic and non-diabetic animals were noted after 30 minutes of hypoglycemia, with increased digging, rearing, movement around the cage, and grooming. At 60 minutes of hypoglycemia, both diabetic and non-diabetic animals became lethargic and reduced movement around the cage. Animals displayed myoclonic jerks in forelimbs and hindlimbs, as well as brief tail dorsiflexion and head nods. At 90 minutes of hypoglycemia, most animals had lost their postural reflex. Sustained tail dorsiflexion (~1-3 sec), head nods, and myoclonic jerks were seen intermittently. Furthermore, and more prominent in the second and third hypoglycemic trials, animals displayed sudden jumps, violent kicking/swimming motions, tail flailing, and barrel rotations.

In terms of seizure severity score, the average for trial one in non-diabetic animals was 1.21±0.22 and 0.97±0.18 in diabetic animals (Fig. 7A). Behavioral seizure severity increased for
both diabetic and non-diabetic animals in trial two, with averages scores of 2.83±0.60 and 2.02±0.47, respectively (p<0.05, Friedman test with Holm-Sidak comparison, Fig. 7A). In trial 3, both groups of animals displayed similar behavioral seizures as was seen in trial 2: nondiabetic animals had a score of 2.83±0.65 and diabetic animals had a score of 3.27±0.54. Both scores were not significant from trial two, but were significantly greater than trial one (p<0.05).

The stated hypothesis was that behavioral seizures will be exacerbated with successive episodes of hypoglycemia and that diabetic animals will exhibit more severe convulsions as compared to non-diabetic animals. Our results confirm that with repeated episodes of hypoglycemia, behavioral seizures become more severe. Both diabetic and non-diabetic animals had significantly higher seizure scores in trial two than trial one, and at least diabetic animals showed a trend for increased seizure severity in trial three as compared with trial two (Fig. 7A).

Interestingly, however, the data shows there was no difference in behavioral severity noted between diabetic and non-diabetic animals in any of the repeated hypoglycemic trials, which is contrary to the hypothesis. This suggests that previous hyperglycemia may not influence seizure behaviors caused by hypoglycemia.

**4.4. Repeated hypoglycemia does not exacerbate blood glucose reductions during hypoglycemia in non-diabetic and diabetic juvenile mice**

Blood glucose levels were measured from samples obtained through the tail-snipping protocol. Animals were fasted for 2 hours prior to first blood withdrawal. Basal blood glucose was 16.7±0.5mM in diabetic animals and 5.3±0.5mM in non-diabetic animals (p<0.001). Within 30
minutes, non-diabetic animals became hypoglycemic at 2.9±0.4mM but diabetic animals were only mildly hypoglycemic at 3.6±0.9mM. At 90 minutes, non-diabetic and diabetic animals had similar glucose levels: 2.4±0.4mM for non-diabetic animals and 2.4±0.2mM for diabetic. 30 minutes following glucose recovery, all animals were restored to euglycemia (5.0±0.2mM for non-diabetic animals and 5.9±0.6mM for diabetic animals; Fig. 7B). In the third hypoglycemic trial, basal blood glucose was 20.2±1.1mM in diabetic animals and 5.1±0.6mM in non-diabetic animals. Diabetic animals basal glucose levels were significantly higher (p<0.05) in trial three than trial one, presumably due to the continued depletion of pancreatic β cells caused by administered STZ after the first trial. At 30 minutes of hypoglycemia, diabetic animals had blood glucose values of 3.4±0.1mM and non-diabetic animals had 3.1±0.4mM. At 90 minutes, diabetic animals were at 2.5±0.4mM and non-diabetic animals were at 2.5±0.3mM.

The stated hypothesis predicted blood glucose levels would decrease with each successive hypoglycemia trial, and that diabetic animals, due to potential HAAF development, will show further reductions in blood glucose during hypoglycemia than non-diabetic animals. The data collected, however, does not support the hypothesis, and blood glucose levels were comparable between diabetic and non-diabetic animals in all trials. The similarity between diabetic and non-diabetic mice suggests that chronic hyperglycemia might not affect blood glucose level reduction caused by exogenous insulin. Secondly, the similarity between the first and third insulin trial suggests antecedent hypoglycemia may not exacerbate blood glucose reductions. Antecedent hypoglycemia, however, did result in more severe behavioral seizures, as was previously presented, and therefore it is possible blood glucose levels do not account for seizure severity.
Figure 7. Behavior and blood glucose during insulin-induced hypoglycemia. A, averaged behavior convulsive scores at 90 minutes of hypoglycemia in single and repeated challenges. *, p<0.05, Friedman test with Holm-Sidak comparison. B, evolution of blood glucose levels during hypoglycemic challenge in 1\textsuperscript{st} and 3\textsuperscript{rd} insulin trials. Basal glucose was taken after 2-hour fast. Blood taken during Recovery occurred 30 minutes after intraperitoneal injection of 1g/kg glucose.
4.5. EEG frequency and amplitude alterations during hypoglycemia in non-diabetic and diabetic animals

Stable simultaneous hippocampal and cortical EEG recordings were achieved in the majority of animals implanted (18 out of 24 [18/24] non-diabetic and 11/12 diabetic mice). Prior to the insulin injection, the EEG revealed rhythmic events in the hippocampus and low amplitude activities in the parietal cortex when the animals were exploring (extended neck, vibrissae movement, rearing) their environment. The hippocampal rhythmic events had a frequency of 7.45 ± 0.21Hz, similar to the mouse hippocampal θ rhythm previously characterized (Buzsáki et al., 2003). When animals were immobile, the hippocampal EEG was dominated by irregular patterns of slow wave activities, and the average frequency of these activities was 3.05 ± 0.15Hz, significantly lower than during the exploratory phase (p<0.001, unpaired t-test). As measured over a 2-minute period, the average amplitude of hippocampal EEG signals was 220±4µV, and the amplitude of corresponding cortical EEG signals was 157±7µV. Diabetic animals similarly showed θ rhythm during exploratory phases (6.83±0.29Hz) and irregular activity in the δ band (3.19±0.11Hz) during immobility, as well as similar amplitudes (hippocampus, 213±10µV; cortex, 148±11µV) (for sample EEG traces and spectral frequencies, see Fig. 8A).

After 30 minutes of hypoglycemia, 18/18 non-diabetic and 10/11 diabetic animals continued to show hippocampal EEG rhythmic events in the θ band (6.45±0.34Hz, non-diabetic; 6.16±0.46Hz, diabetic) and irregular activities (3.84±0.41Hz, non-diabetic; 2.97±0.38, diabetic). The mean amplitudes of hippocampal EEG signals were 281±8µV (non-diabetic) and 272±15µV (diabetic), while cortical EEG signals were 201±10µV (non-diabetic) and 198±12µV (diabetic), all of which were significantly greater than baseline recordings (p<0.001, paired t-test).
After 60 minutes of hypoglycemia, 8/18 non-diabetic mice and 3/11 diabetic mice continued to show θ-like hippocampal rhythmic activities (6.70 ± 0.40Hz, non-diabetic; 6.89±0.10Hz, diabetic), and hippocampal slow wave activity was prominent in all animals recorded (2.39 ± 0.22Hz, non-diabetic; 2.14±0.18Hz, diabetic). The averaged amplitudes of hippocampal and cortical EEG signals in non-diabetic animals were 208 ± 4µV and 135 ± 12µV, while in diabetic animals, it was 179±12µV and 128±7µV, respectively, which was significantly decreased from baseline recordings (p<0.001, paired t-test).

At 90 minutes of hypoglycemia, θ-like activity was present in only 1 non-diabetic animal and completely absent in diabetic animals. The prominent EEG pattern was slow, low-amplitude activity of 1.53±0.13Hz (non-diabetic) and 1.52±0.20Hz (diabetic). Overall, the hippocampal and cortical EEG signals were further diminished from baseline values, and their mean amplitudes were 154±6µV and 117±12µV respectively in non-diabetic animals (p<0.001, paired t-test). Diabetic animals had similar cortical EEG signal amplitudes (116±11µV) but significantly reduced hippocampal signals (118±14µV). This pattern was observed when taking the average of amplitudes for all 3 insulin trials (p<0.05; Fig. 8B).

After *i.p.* administration of glucose, the hippocampal and cortical EEG activities in both non-diabetic and diabetic animals gradually increased in amplitude and hippocampal θ-like rhythmic events were noticeable in 10/18 non-diabetic and 4/11 diabetic mice 30 minutes after the glucose rescue (6.75±0.26Hz for non-diabetic, 6.23±0.30Hz for diabetic) but amplitudes from cortical (126±4µV non-diabetic; 123±12µV diabetic) and hippocampal (163±3µV non-diabetic; 137±14µV diabetic) EEG remained significantly decreased from basal recordings (p<0.001, paired t-test; for EEG sample traces and spectral frequencies, see Fig. 8A). All data on
prominent EEG frequencies and signal amplitudes in the hippocampus and cortex of both non-diabetic and diabetic animals undergoing repeated hypoglycemic episodes can be found summarized in Table 1.

These results echoed findings seen in previous studies examining adult animals (Auer, 2006; Suh et al., 2003; Velísek et al., 2008): initial responses to hypoglycemia include increased amplitudes and decreased signals, while prolonged hypoglycemia eventually brought about reduced amplitude and frequency to the point of isoelectricity. This study focused upon the behavior of rhythmic activity in the θ region, which is seen mostly during exploratory animal behavior (Buzsáki et al., 2003). As hypoglycemia has been associated with cognitive impairments in young children (Davis et al., 1997), it was hypothesized that θ-like rhythms would disappear during hypoglycemic episodes. Indeed, at 90 minutes of hypoglycemia, θ-like rhythms were not present in any diabetic animals and in a significant majority (17/18) of non-diabetic animals. Such rhythms, however, eventually returned and were not altered, even after multiple hypoglycemic episodes. The θ rhythm is known to be robust (Siegel et al., 2000) and perhaps more severe and frequent hypoglycemic challenges may produce different results.
Figure 8. EEG during hypoglycemia. A, left side shows representative EEG traces collected from a non-diabetic mouse before and at different times following an intra-peritoneal injection of insulin (20UI/kg). "Recovery" refers to EEG traces recorded 30 minutes after an intra-peritoneal injection of glucose (1g/kg). Right side shows a spectral frequency plot of prominent activity in the hippocampal EEG during active (mobile) and inactive (immobile) behavioral phases. B, comparison of averaged hippocampal and cortical EEG signal amplitudes at 90 minutes of hypoglycemia between saline, non-diabetic, and diabetic animals. *, p<0.05. ANOVA.
Table 1. Prominent frequencies and amplitudes in hippocampal and cortical EEG signals in non-diabetic and diabetic mice during repeated hypoglycemic episodes. Data for first insulin trial presented in RESULTS (4.5.). Asterisks represent significant differences as compared to “Basal” values (*, p<0.05; **, p<0.01). Hipp., hippocampus.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time (min)</th>
<th>Non-diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hipp. Frequency (Hz)</td>
<td>Amplitude (µV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Θ rhythm</td>
<td>Slow wave</td>
</tr>
<tr>
<td>2</td>
<td>Basal</td>
<td>7.02±0.43</td>
<td>2.05±0.17</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>6.81±0.37</td>
<td>2.69±0.46</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>6.99±0.28</td>
<td>2.35±0.60</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>-</td>
<td>2.13±0.14</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>6.31±0.36</td>
<td>2.32±0.24</td>
</tr>
<tr>
<td>3</td>
<td>Basal</td>
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<td>2.01±0.37</td>
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<tr>
<td></td>
<td>30</td>
<td>7.13±0.50</td>
<td>2.45±0.29</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>6.73±0.33</td>
<td>2.31±0.42</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>-</td>
<td>2.06±0.39</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>6.28±0.83</td>
<td>1.89±0.11</td>
</tr>
</tbody>
</table>
4.6. Repeated hypoglycemic episodes caused the emergence of interictal discharges in hippocampal and cortical EEG

After 90 minutes of hypoglycemia, all mice exhibited severely depressed EEG activity (as discussed in the previous section). However, 2/18 non-diabetic animals (none of the diabetic mice) displayed sudden rhythmic discharges from both the cortex and the hippocampus. In the second trial, 14 non-diabetic and 9 diabetic animals showed similar interictal discharges in the hippocampus and cortex, and in the third trial, 17 non-diabetic and 9 diabetic animals displayed this phenomenon (for EEG sample trace, see Fig. 9). A summary of these interictal pattern characteristics can be found in Table 2. These rhythmic activities are distinct from the physiological hippocampal θ rhythms because the former were not associated with exploratory behaviors. Nonetheless, these events were often (~80%), but not always, associated with body jerks, myoclonus, or tail dorsiflexion (Fig. 9).

The stated hypothesis was that hypoglycemic seizures would induce ictal discharges in both the hippocampal and cortical EEG. The results do not show clear ictal discharges, but it is possible this rhythmic activity may be a low-amplitude “epileptiform discharge” resulting from hypoglycemic challenge. Interestingly, more animals experienced these rhythmic events with repeated hypoglycemia (Table 2).

Furthermore, the hypothesis predicted the diabetic condition and repeated hypoglycemia would each exacerbate abnormal EEG events. Our results do not support the idea that diabetes exacerbates the EEG during hypoglycemia, but repeated hypoglycemia did increase the duration of the rhythmic events seen in both groups (p<0.01; Table 2). Similarly, there was a trend for the
increase in frequency of such events in both diabetic and non-diabetic animals. These data, hence, may indicate repeated hypoglycemic episodes may have a pro-electrographic seizure effect in the hippocampus and cortex.

**Figure 9.** Interictal discharges in EEG during 90 minutes of hypoglycemia. Representative EEG traces from non-diabetic and diabetic animals during the third insulin challenge, with grey boxes depicting instances of interictal discharge. An image represents the behavioral state of a diabetic animal (lethargic with tail dorsiflexion) during an interictal discharge episodes. *, electrical artifact due to violent behavioral convulsion preceeding interictal discharge.
Table 2. Characteristics of epileptiform rhythmic discharges observed in recurrent hypoglycemic animals. Number of incidents was calculated using only animals that showed epileptiform discharges. *, p<0.01.

<table>
<thead>
<tr>
<th>Insulin Trial</th>
<th>Non-diabetic (n=18)</th>
<th>Diabetic (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animals with epileptiform activity</td>
<td>Incidents (episodes per animal)</td>
</tr>
<tr>
<td>1</td>
<td>3.88±0.67</td>
<td>4.2±0.52</td>
</tr>
<tr>
<td>2</td>
<td>6.42±0.35*</td>
<td>6.04±0.02</td>
</tr>
<tr>
<td>3</td>
<td>7.81±0.79</td>
<td>8.25±1.40</td>
</tr>
<tr>
<td>1</td>
<td>4.79±0.64</td>
<td>6.90±0.64</td>
</tr>
<tr>
<td>2</td>
<td>4.85±2.23</td>
<td>6.79±3.90</td>
</tr>
<tr>
<td>3</td>
<td>4.85±2.23</td>
<td>6.79±3.90</td>
</tr>
</tbody>
</table>
Chapter 5
Discussion

5.1. Re-examination of Objective and Hypotheses

The objective of this thesis was two-fold: (1) to describe and compare cortical and hippocampal EEG evolution in single and repeated hypoglycemic episodes in juvenile diabetic and non-diabetic mice, and (2) to describe and compare the behavioral seizure severity in repeated hypoglycemia between juvenile diabetic and non-diabetic mice. The main hypothesis was that repeated hypoglycemic episodes would promote EEG and behavioral seizures during hypoglycemia, and that diabetic animals would show exacerbation of these abnormalities in comparison to non-diabetic animals.

Collectively, the results gathered partially support the hypothesis in that repeated hypoglycemic episodes caused increased behavioral seizure severity and induced transient rhythmic events in the hippocampal and cortical EEG. Surprisingly, however, there was a lack of difference between diabetic and non-diabetic animals in terms of behavioral seizure severity and EEG abnormalities. One difference noted in these experiments was that hypoglycemia consistently caused a significant reduction in hippocampal EEG activity at 90 minutes of hypoglycemia, as compared with non-diabetic animals.
(1) Insulin-induced hypoglycemia will trigger behavioral seizures more frequently in juvenile mice in comparison with adult mice. This will correlate with clinical findings that consistently report young diabetic children as more prone to seizure development than adults.

The results support this hypothesis. 21-day old juvenile mice showed greater frequency of behavioral seizure than 10-month old adult mice given weight-adjusted equivalent doses of insulin.

As was previously discussed, this finding concurs with evidence shown in clinical (DCCT, 1993; 2007) and in vitro preparations (Abdelmalik et al., 2007) that younger age correlates with greater susceptibility to hypoglycemic seizures.

The increased susceptibility of juvenile mice to hypoglycemic seizures can be attributed to a number of reasons. Buckner and Biesold used the light microscope periodic acid-Schiff method to study glycogen content in rat brain, concluding that greater glycogen is found in the developing brain than in adult brains. As seizures are a hypermetabolic phenomenon, the greater amount of stored glucose in juvenile animals may facilitate hypoglycemic seizures more so in the young rather than adults.

Additionally, immature animal brains require greater glucose supply than in adults to facilitate growth and development (Clarke and Sokoloff, 1999), which may mean immature brains are more prone to negative consequences (such as seizures) in hypoglycemic situations than adult brains.
Furthermore, a study by Khan et al. (1999) revealed there is an upregulation of GLUT1 and GLUT3 transporters in the murine brain between postnatal day 1 to 60, resulting in enhanced brain glucose uptake. Thus, during hypoglycemia, juvenile animals may be less able to extract necessary amounts of glucose from circulation for brain maintenance.

(2) **During recurrent hypoglycemia, each hypoglycemic episode will increase the severity of hypoglycemic behavioral seizures.** Diabetic animals, which are known to have aberrant glucose counter-regulation, will show further exacerbated behavioral seizures compared with non-diabetic animals.

The results partially supported this hypothesis. Repeated hypoglycemic seizures were shown to exacerbate behavioral seizures in both diabetic and non-diabetic animals. However, no significant difference in seizure severity was noticed between diabetic and non-diabetic animals during any of the insulin trials.

Impairments in the glucose counter-regulatory system caused by diabetes and repeated hypoglycemia form the basis of this hypothesis. Chronically elevated glucose levels and recent antecedent hypoglycemia are thought to, through different mechanisms, desensitize glucose sensing cells, which in turn reduces sympathoadrenal responses to hypoglycemia (Cryer, 2008). The result is unawareness of hypoglycemia and an increased susceptibility to severe hypoglycemia and related behavioral characteristics (such as seizures).

The finding that repeated hypoglycemic episodes contribute to heightened seizure severity appears to support this theory, but further investigation, including time-course studies of glucose hormone concentrations in the blood during repeated hypoglycemic challenges, is needed.
The lack of difference of seizure severity between diabetic and non-diabetic animals may suggest that the effects of diabetes and repeated hypoglycemia on glucose counter-regulation are not additive. Alternatively, perhaps a longer duration of diabetes prior to hypoglycemic episodes is required to observe changes in seizure severity.

(3) **Repeated hypoglycemic episodes will worsen hypoglycemic-induced reductions in blood glucose, possibly due to impaired glucose counter-regulation.**

The results do not provide support for this hypothesis. Blood glucose levels during the first and third insulin trials were not statistically different in both diabetic and non-diabetic animals. Furthermore, diabetic and non-diabetic animals had similar blood glucose levels in all insulin trials.

This finding is at odds with other presented results. The assumption that repeated hypoglycemia caused behavioral and EEG changes by adversely affecting glucose counter-regulation, thereby causing changes in blood glucose levels, may not be accurate. Another possibility is that repeated hypoglycemia may cause a “rewiring” of brain electrical circuits, independent of blood glucose concentrations, which promote seizure-like (both electrographic and behavioral) activity.

(4) **During hypoglycemia, the hippocampal θ rhythm will transiently disappear.**

Alterations in the θ rhythm will be present after repeated hypoglycemia. This hypothesis is based upon the finding that hypoglycemia affects cognitive functions and the hippocampal θ wave is associated with exploratory behavior.
The results did not fully support this hypothesis. Hippocampal θ-like rhythms were absent in almost all diabetic and non-diabetic animals in the later stages of hypoglycemia. However, these rhythms eventually returned proceeding each hypoglycemic challenge and were not altered in frequency or amplitude.

Interpreting that the θ wave was directly affected during hypoglycemic episodes must be done with caution. For example, it can not be discounted that, because of overall lethargy seen in the later stages of hypoglycemia, the loss of the θ wave was merely due to the loss of exploratory behavior.

(5) **Behavioral seizures will correlate with electrographic seizures in the hippocampal and cortical EEG electrodes. Repeated hypoglycemia will exacerbate these ictal discharges by increasing their frequency and duration. Diabetic animals will show greater occurrences of hypoglycemic seizures than non-diabetic animals.**

The results partially support this hypothesis. Classical electrographic seizures were not observed in any of the insulin trials in either diabetic or non-diabetic animals. However, repeated hypoglycemia was associated with the emergence of transient rhythmic events in both the hippocampus and cortex. Repeated hypoglycemia caused an increase in the number of animals exhibiting this phenomenon, as well as increasing their duration.

The rhythmic events seen during repeated hypoglycemia have not been reported elsewhere. Elucidation of their mechanism and origin is beyond the scope of this work. Of interest, however, is the fact that behavioral seizures occurred with and without the presence of EEG correlates in the hippocampus and cortex. Del Campo (2009) noticed the same peculiarity when
examining hypoglycemic rats, and suggested deeper brain structures be monitored. It is possible that hypoglycemic EEG abnormalities originate in deeper brain structures and, through repeated hypoglycemic challenges, recruit additional brain structures and become a more global phenomenon. The hippocampus and cortex may, hence, be secondary structures in the pathogenesis of seizures. Further work (see DISCUSSION 5.5) will be needed to verify this possibility.

### 5.2. Relation of Findings to Literature

Hypoglycemia, nearly a century after its clinical documentation, remains a significant barrier to glycemic control in diabetes. The brain is a structure particularly vulnerable to hypoglycemia, due to its continuous dependence upon circulating glucose for energy. A variety of animal models have been established to examine a multitude of effects hypoglycemia has upon the brain, including: neurodegeneration (Auer et al., 1984; Suh et al., 2003), cognitive impairment (McNay et al., 2004; 2006); seizure development (Abdelmalik et al., 2007; Velišková et al., 2008), and EEG abnormalities (Del Campo et al., 2009).

What is lacking in this area of research is the use of an animal model representative of young diabetic children, who are repeatedly shown to be most susceptible to hypoglycemia and related consequences (Danne et al., 1994; Davis et al., 1997; DCCT, 1993; Hershey et al., 2005; Schultz et al., 1999). Aside from in vitro work on immature (8-13 days old) mice by Abdelmalik et al. (2007), previous studies utilized adult animals for investigating hypoglycemia. Differences between juvenile and adult mice have been documented in terms of effect of stress (Stone and Quartermain, 1998), antidepressant treatment (Mason et al., 2009), and adhesion molecule
expression (Ching et al., 2007). Also, metabolic differences between juvenile and adult brains, such as suggestions that there are larger stores of glycogen (Bruckner and Biesold, 1981; Edwards and Rogers, 1972, Margolis et al., 1976) may play a role in the differential reactions to hypoglycemia. This thesis has attempted to establish a juvenile mouse model, and use it to correlate behavioral and electroencephalographic responses to repeated hypoglycemia, within and without the context of type 1 diabetes.

With respect to behavioral and EEG responses to repeated hypoglycemia, the experimental results has shown surprisingly little difference between non-diabetic and diabetic animals. It is possible that diabetes may not directly exacerbate neurophysiological abnormalities caused by hypoglycemia. In humans, Tallroth and colleagues examined the EEG profile of type 1 diabetic and non-diabetic men, concluding that in both groups there was an equal increase in low-frequency activity and marked reduction of evoked potential (P300) amplitudes.

The one difference noted was that hippocampal EEG signal amplitude was significantly reduced in diabetic animals, as compared with non-diabetic animals, during the later stages of hypoglycemia. EEG signals are the summation of neuronal synaptic potentials (Kandel, 2000), hence a reduction in the signal could signify a reduced number of active neurons.

The exacerbation of behavioral seizures, correlated with the emergence of transient rhythmic activity, following repeated hypoglycemia provides an avenue for further investigation. It is possible these events are precursors for classic electroencephalographic seizures. Additionally, since such events were seen more frequently and with longer durations after repeated hypoglycemia, it may be that multiple challenges promote seizure generation. Further study is
required to test these ideas. At least one study (Velišková et al., 2008), however, does show hypoglycemic seizures after a single episode of hypoglycemia, yet the generation of seizures was inconsistent in non-fasted animals and it required the use of a high (30UI/kg) doses of insulin. Suh and colleagues (2003) used a lesser dosage of insulin (15UI/kg) in rats and remarked that seizure generation was rare after a single insulin challenge, with only 2/26 animals showing electroencephalographic seizures. Hence, the data in this thesis suggests a paradigm involving multiple hypoglycemic challenges for the investigation of hypoglycemic seizure origins and mechanisms.

Investigation of the hippocampal rhythms revealed that hypoglycemia was associated with a transient disappearance of rhythmic events in the θ band. It is possible that the loss of the θ activity is simply due to the reduction of exploratory activity associated with hypoglycemia, however in vitro work by Rabinovici and colleagues (2000) showed evoked θ frequency micro-EEG oscillations in rat brain slices progressively declined during glucose deprivation. Because θ waves have been associated with learning and memory (Buzsáki, 2005), hypoglycemia-induced loss of this rhythm may imply cognitive impairments. McNay et al. has published two studies (2004; 2006) which examine the effects of recurrent hypoglycemia upon cognition in adult rats. They have interestingly revealed that recurrently hypoglycemic rats had cognitive impairments, but only under hypoglycemic, and not euglycemic, conditions.

The results of this thesis suggest episodes of hypoglycemia may have an additive effect in exacerbating both behavioral and electroencephalographic abnormalities associated with hypoglycemia. From a clinical perspective, this may be of value when assessing risk for hypoglycemic seizures. Usually, diabetic patients are given increasing amounts of insulin, so as
to gain greater glycemic control, up until adverse reactions to hypoglycemia become noticed (Cryer, 2006). The data from this thesis may suggest those with a previous history of hypoglycemia are at particular risk for the development of hypoglycemic seizures.

5.3. Novelty and Significance

Although a few studies already exist examining hypoglycemia in animals from behavioral (Velísek et al., 2008) and EEG (Kirchner et al., 2006; Velíšková et al., 2007) perspectives, to our knowledge no study currently examines EEG changes in diabetic and non-diabetic animals over the course of repeated hypoglycemia. Furthermore, studies examining hypoglycemia fail to include juvenile animals in their methodology.

A few studies have attempted to describe the evolution of EEG during hypoglycemia, however descriptions have been vague (Auer et al., 1984; Suh et al., 2003) and without significant quantitative data. This thesis provides detailed hippocampal frequency and hippocampal/cortical amplitude data throughout the duration of repeated hypoglycemic challenges in both diabetic and non-diabetic animals.

The results have supported the stated hypothesis that repeated episodes of hypoglycemia exacerbate abnormalities in behavior and EEG signals seen during hypoglycemia. Results from the DCCT (2007) have suggested the higher prevalence of hypoglycemia in diabetic adults using intensive insulin therapy (3 or more administrations per day) does not warrant a switch to conventional insulin therapy (1 or 2 administrations per day). This study, which has focused upon juvenile animals, has shown significant exacerbation of hypoglycemic-induced symptoms
in juvenile mice after repeated hypoglycemic episodes. If applied to the clinical setting, these results may suggest a re-examination of the threat of repeated hypoglycemia specifically to diabetic children. Those presenting high recurrence rates for hypoglycemia may require modifications in their diabetic treatment regimen, even if diabetic adults with the same symptoms may be recommended to continue with the same treatment.

5.4. Potential Pitfalls and Limitations

(1) Potential effect of implantation on response to hypoglycemia

As was discussed in RESULTS 4.3., implanted juvenile mice did not show behavioral seizures with the same consistency as non-implanted mice, despite identical hypoglycemia induction protocols. It is possible that surgical implantation, *per se*, may have affected the brain and its response/symptoms to hypoglycemic challenges.

Implantation involved the insertion of one 0.3mm-wide recording electrode directly into the animal’s right hippocampus and two similar electrodes into the left parietal cortex. Although some neurodegeneration is expected directly at the site of implantation, staining with Fluoro-Jade showed that brain damage was minimal (data not shown). From a visual perspective, experimenters found implanted and non-implanted mice were behaviorally indistinguishable. Finally, the practice of recording intracranial EEG in animals models in not novel, with previous studies (Del Campo et al., 2009; Velíšková et al., 2008, Wu et al., 2008) already demonstrating its effectiveness.
By increasing dosage from 15UI/kg to 20UI/kg, and using a faster acting insulin type, seizure behavior became more pronounced, which suggests that, if electrode implantation has any effect on the brain’s reaction to hypoglycemia, it is probably anti-convulsive. Without performing a series of neurophysiological/cognitive tests proceeding implantation, an understanding of the effects electrode implantation has on the brain escapes elucidation, and therefore, presents a limitation in these experiments.

(2) Experimental parameters

The experiments of this thesis attempt to mimic hypoglycemia observed in the clinical setting, and so experimental parameters (such as frequency and duration of hypoglycemia, insulin dosage, and hypoglycemia severity) were selected accordingly.

Repeated hypoglycemic episodes were performed one week apart, similar to the protocol utilized by McNay et al., (2006) in rats. An earlier study by McNay and Sherwin (2004) attempted daily hypoglycemia in rats, but it was noted that this did not properly reflect clinical hypoglycemia, hence the follow-up study in 2006. According to Cryer (2008), however, diabetic humans experience symptomatic episodes of hypoglycemia twice per week on average. Thus, the choice of interval between hypoglycemic episodes is debatable.

Similarly, duration of hypoglycemic episodes can be contested. The decision to use a maximum of 90 minutes was based upon preliminary experiments in non-implanted animals, where most animals developed severe behavioral seizures and required glucose rescue at approximately this time frame. In most other studies, a longer duration is selected: McNay et al., (2004; 2006) used a 3-hour period of hypoglycemia; Del Campo et al. (2009) recorded until animal death (~3.5
hours); and Velíšková et al., (2008) also recorded for 4 hours or more. All the aforementioned studies were, however, performed in rats, hence it is possible differences in basal metabolism between rats and mice account for the time intervals required for hypoglycemia induction. Anuradhna et al. (2004) examined hypoglycemia in mice (albeit without EEG recordings) and chose a maximum of 2 hours for hypoglycemia, which is more similar to this thesis.

The chosen insulin dosage (20UI/kg) produced only moderate hypoglycemia (above 2mM). Moderate hypoglycemia is more common in clinical diabetes than severe hypoglycemia (Cryer, 1997), but tabulating frequencies of moderate hypoglycemia is difficult (Strachan, 2007). Although this approach mimics clinical hypoglycemia, it may not be the best approach to study more severe symptoms of hypoglycemia, such as seizures. Velíšková and colleagues (2008) examined hypoglycemic seizures in rats, and noticed that at the first seizure, peripheral blood glucose levels were at about 1.1mM.

Finally, the duration of diabetes preceding insulin trials can also be debated. Only 3 days elapse between confirmation of hyperglycemia in STZ-treated animals and the onset of insulin challenges. This may not be sufficient time for neurophysiological changes resulting from hyperglycemia to emerge and, hence, a potential reason why this thesis found few differences in behavioral and EEG profiles between STZ-treated and naïve animals. Selecting younger age at which to commence the STZ-induction protocol in animals may help address this limitation.

(3) Applicability to humans

As is true in all animal models, correlation of experimental findings to the human population must be done with caution. Nonetheless, animal models have played an important role in
understanding neuronal mechanisms responsible for EEG interpretation in both physiological and pathological settings (Engel and Schwartzkroin, 2006). Hence the value of animals models can not be discounted.

One drawback, however, in the applicability to humans is that the diabetic animals generated in this study were allowed to remain chronically hyperglycemic until administration of insulin for hypoglycemic challenge. In the clinical setting, daily insulin therapy prevents extended periods of hyperglycemia. Furthermore, there is evidence that hyperglycemia can influence seizure thresholds (Schwechter et al, 2003). This adds complexity in the interpretation of seizure susceptibility, and warrants further investigation using diabetic mice that are given daily insulin therapy.

(4) Electroencephalographic seizures

The lack of classic electroencephalographic seizures may be due to a variety of reasons, including limitations in the experimental protocol (as previously discussed). It is possible, however, that during severe behavioral seizures (such as those seen at 90 minutes of hypoglycemia), artifacts masked EEG signals. The transient rhythmic events observed in this study were usually associated with minor behavioral seizing, such as tail dorsiflexion and brief myoclonus. True ictal discharges may possibly occur during severe behavioral convulsions. Overcoming this obstacle may require alternative placement of the grounding electrode or modification of the electrode assembly. Alternatively, use of a temporary paralytic agent (such as D-tubocurare) or the gentle restraint of an animal during severe hypoglycemia may rectify this issue.
5.5. Future Directions

(1) Assessing cognitive function in juvenile diabetic and non-diabetic animals

The results of this thesis show loss of hippocampal rhythms in the θ frequency band. Furthermore, diabetic animals had markedly reduced hippocampal EEG signals compared to non-diabetic animals during prolonged hypoglycemia. Combined with clinical reports on cognitive impairment in diabetic children (INTRODUCTION 1.4.2.2.), investigation of the memory-related tasks in diabetic juvenile animals is a plausible avenue to follow.

Juvenile mice will be divided into diabetic and non-diabetic groups. Cognitive tests, such as the Morris water maze and novel object recognition task, could be used. The effect of a single antecedent episode of hypoglycemia versus repeated ancetedent episodes of hypoglycemia could be tested. A preliminary investigation of the effects of repeated hypoglycemia in non-diabetic animals performance in the novel object recognition task did not reveal any impairments (APPENDIX A).

(2) EEG recording of deeper brain structures during repeated hypoglycemia

Recordings of multiple brain regions are essential to determine the focus and propagation of hypoglycemic seizures. The results in this thesis indicate that only after multiple episodes of hypoglycemia do abnormal rhythmic events emerge in the hippocampus and cortex, despite behavioral seizures being present in all challenges. This suggests the hippocampus and cortex may be recruited into the seizure mechanism and that the origin of hypoglycemic seizures occurs in other brain structures. Targeting deeper brain structures may provide additional clues to the pathophysiology of hypoglycemic seizures.
(3) *In vitro* experimentation using recurrently hypoglycemic diabetic animals

Abdelmalik et al. (2007) has already characterized the seizure susceptibility and pharmacology of immature intact hippocampi to glucose deprivation. To further this work, it may be of interest to examine seizure susceptibility in glucose deprivation using an intact juvenile diabetic mouse hippocampus. Also, a comparison of diabetic and recurrently hypoglycemic diabetic hippocampi will further shed light on the abnormal neuronal activities within the diabetic brain.
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Appendices

A: Preliminary results of novel object recognition task on repeatedly hypoglycemic non-diabetic animals

Figure A. Novel Object Recognition (NOR) task. A, amount of time spent investigating novel object vs. familiar one compared between repeatedly hypoglycemic animals and repeated saline injected (control) animals. * p<0.05, ANOVA. B, image of objects used during novel object recognition task.
**B: Preliminary in vitro investigation of sharp waves (SPWs) in repeatedly hypoglycemic non-diabetic animals**

**Figure B.** *In vitro* characteristics of recurrent insulin-challenged animals. *A*, left side shows spontaneous sharp waves (SPWs) seen in various regions of hippocampal slice. Right side trace compares SPW onset between CA3, CA1, and DG. *B*, high-frequency stimulation (HFS, 80Hz for 1s) induces transient SPWs in CA3 and CA1. *C*, table summarizing characteristics of SPWs in recurrent insulin- and saline-challenged animals.
C: In vitro inter-ictal field discharges in hippocampal tissue during low glucose perfusion

Figure C. Examination of low glucose ACSF upon regional initiation of spontaneous field discharges. A, spontaneous activity from the CA1, CA3, and DG was observed with 5mM glucose and 0.5mM glucose ACSF perfusion. B, enlarged view of spontaneous field discharges from CA3 region. Regional onset of interictial spontaneous field discharges was observed between the CA3 and DG (C) and the CA3 and CA1 (D).