University of Alberta

The Effect of Hypoglycemia on the Functional and Pathological Outcome of the Newborn Rat

ALIREZA KARIMI POUR

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Examining Committee

Dr. Jerome Y. Yager, Pediatrics, Supervisor

Dr. Po-Yin Cheung, Pediatrics, Thesis Committee Member

Dr. Bernard Thébaud, Pediatrics, Thesis Committee Member

Dr. Chris Hanstock, Biomedical Engineering, Thesis Committee Member

DEDICATION

I dedicate my thesis to

my mother and father who help make my life whole,

my lovely siblings who have a very special place in my heart,

and

To my teachers, lifetime partners in my learning.

THESIS ABSTRACT

Controversy remains about the contribution of hypoglycemia to brain damage in the newborn. Therefore, the objective of this study was to determine the effects of *isolated* hypoglycemia on damage to the immature rat brain. Seven-day-old rats, equivalent to a late preterm human newborn, were placed in either Sham or hypoglycemic groups. Hypoglycemia was induced by insulin infusion for variable periods of time. Outcomes were assessed by behavioral, neurochemical and neuropathologic determination. Rats were categorized as having mild, moderate, or severe hypoglycemia. Behavioral tests revealed no abnormality in hypoglycemic animals. Floro-JadeB showed significant damage in the thalamic reticular nucleus (TRN) of the severe hypoglycemic animals at PD9. However, neuronal (Neu-N), astrocytic (GFAP), and myelin (MBP) staining at PD21 showed no brain injury. There was a significant rise in aspartate and arginine, and drop in glutamine and alanine of hypoglycemic brains. Oxidative stress markers were also increased in hypoglycemic brains. We conclude that isolated prolonged severe hypoglycemia caused a transient, region specific increase in neuronal cell death within the TRN. Though transient in nature, the associated neurochemical alterations warrant further research to determine if more subtle long-term effects may result.

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LIST OF ABBREVIATIONS

AGA	Appropriate for Gestational Age
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
CBF	Cerebral Blood Flow
CGU	Cerebral Glucose Utilization
CMR	Cerebral Metabolic Rate
dl	deciliter
ECM	Extracellular Matrix
EAA	Excitatory Amino Acids
F	Female
FJB	Fluoro-Jade-B
GFAP	Glial Fibrillary Acidic Protein
H&E	Hematoxylin and Eosin
h (hr)	Hour
HI	Hypoxia-Ischemia
IUGR	Intrauterine Growth Retardation
L	litter
LGA	Large for Gestational Age
M	Male

MBP	Myelin Basic Protein
mg	milligram
mmol	millimole
MMP	Matrix Metalloproteinase
NADPH	reduced form of NADP+ (nicotinamide adenine dinucleotide phosphate)
Neonatal	The time period from birth through the first 4 weeks after birth
Neu-N	Neuronal Nuclei
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NS	Normal Saline
PD	Postnatal Day
Perinatal	The time period spanning shortly before and after birth
Postnatal	The time period following birth
Prenatal	Period of time spanning conception to the beginning of labor
rCBF	regional Cerebral Blood Flow
ROS	Reactive Oxygen Species
s (sec)	second
SGA	Small for Gestational Age
TRN	Thalamic Reticular Nucleus
°C	Degrees Centigrade

Background

Introduction

A developing immature brain requires an adequate amount of metabolizable substrate to maintain normal function and development during the perinatal period. Glucose is the principal energy substrate for both the adult and immature brain under normal physiologic conditions, providing more than 90% of brain energy requirement (Hernandez, Vannucci et al. 1980). However, under some physiological or pathological conditions of the brain, glucose may be unavailable. If this occurs, there are mechanisms through which the human brain tries to compensate for the shortage of glucose during the perinatal period. For instance, when the demand for energy production is superseded, alternative substrates, such as lactate and ketone bodies, can be made available to supplement glucose (Nehlig 1996; Vannucci and Vannucci 2000).

Transient hypoglycemia is common in the neonatal period and is considered a normal feature of adaptation from intrauterine to extra-uterine life (Williams 2005). Severe, recurrent and/or persistent hypoglycemia may also occur within the first days of life, often associated with other physiologic and pathophysiologic alterations facing the newborn infant. Some of these conditions include prematurity and intrauterine growth restriction, but a term infant may also be compromised by challenges to metabolic stability such as sepsis, hypoxia-ischemia, or seizures (Williams 2005).

Despite awareness of the crucial need for glucose in cerebral energy metabolism, particularly during the complex transition from the fetal period to newborn life, questions still remain about the effect of hypoglycemia, *per se*, on brain damage, and its contribution to neurodevelopmental outcome. Even though the immature brain is more resistant to hypoglycemia than adults (described later), several studies indicate that hypoglycemia in the immature brain may lead to permanent cerebral damage (Volpe 2001). In studies on the human neonate, magnetic resonance imaging (MRI) findings have indicated a specific pattern of abnormality, felt to be caused by hypoglycemia. In this regard, areas of white matter attenuation have been seen, particularly in parieto-occipital brain regions (Pildes, Cornblath et al. 1974; Duvanel, Fawer et al. 1999; Alkalay, Flores-Sarnat et al. 2005; Burns, Rutherford et al. 2008). Unfortunately, these studies were unable to assess the effect of isolated hypoglycemia on the immature brain, in the absence of other prenatal and/or perinatal perturbations. None-the-less, based on this information, recommendations have been made to carefully monitor and treat hypoglycemia in these infants to prevent the detrimental neurological and developmental outcomes.

Given that the contribution of hypoglycemia *per se,* to cerebral injury remains unknown, it is the intent of my research proposal to study the neurodevelopmental and structural alterations of the immature rat brain following mild to severe *isolated* hypoglycemia.

Definitions

There is no precise definition of neonatal hypoglycemia. The difficulty surrounding a precise definition arises from the dynamic nature of glucose metabolism during early development and its alteration according to the needs of the infant and his/her surrounding environment. Neonatal hypoglycemia varies depending on a host of factors including the presence or absence of neurologic symptoms, gestational age of newborn, the age of the infant following birth, birth weight, duration of low blood sugar, and a host of ancillary factors, including pathophysiologic contributions from hypoxia-ischemia, seizures, or sepsis as might occur in the newborn infant (Yager 2002; Alkalay, Flores-Sarnat et al. 2006).

Serial plasma glucose measurements in term, healthy newborns are shown in Table 1. Within the first two hours of life there is an initial drop to 3.05 mmol/l, followed by a rise to 3.88 mmol/l from 3 to 72 hours, and levels in excess of 4.44 mmol/l beyond the third day (Srinivasan, Pildes et al. 1986). Heck et al. defined term neonatal hypoglycemia as plasma glucose concentrations < 1.7 mmol/l within the first 24 hours of life and < 2.2 mmol/l between 24 and 48 hours (Heck and Erenberg 1987).

In 1937, Hartmann and Jaudon published a study of 286 neonates and infants with significant hypoglycemia as determined by persistently low blood sugars and the presence of clinical manifestations. In their studies, hypoglycemia was defined as ‘mild’ (2.2 – 2.8 mmol/l), ‘moderate’ (1.1 to 2.2 mmol/l) or ‘extreme’ (<1.1 mmol/l) (Hartmann 1937). In a study

by Koh et al. (Koh, Eyre et al. 1988), that surveyed a considerable number of pediatric textbooks and consultants regarding their definition of neonatal hypoglycemia, definitions varied from <1mmol/l to <4mmol/l in term healthy newborn infants. In many textbooks neonatal hypoglycemia is defined as < 2.2 – 2.5 mmol/L in full term and < 2.6 – 2.8 mmol/L in preterm infants (Table 1) (Cryer 2008).

Table 1. Suggested Definitions of Neonatal Hypoglycemia

Age (hours from birth)	*Plasma Glucose Level (mmol/L)*
0-3	< 1.94
3-24	< 2.22
> 24	< 2.5
Srinivasan et al. J Pediatr 1986; 109: 114-117.	
0-24	< 1.7
24-48	< 2.2
Heck LJ. J Pediatr 1987; 110: 119-122	

Infant	*Blood Glucose Level(mmol/L)*
Full term	< 2.2-2.5
Preterm	< 2.6-2.8
(Lucas, Morley et al. 1988; Cryer 2008)	

Since the definition of neonatal hypoglycemia is controversial, an ‘‘Operational Threshold’’ has been defined by Cornblath et al. as the concentration of blood or plasma glucose at which clinicians should consider intervention (Cornblath, Nakamura et al. 1997; Cornblath, Hawdon et al. 2000; Cornblath and Ichord 2000). This threshold varies

depending on different clinical factors. According to their findings, any infant with clinical manifestations of hypoglycemia should be tested, and intervention should be taken for those with values less than 45 mg/dl (2.5 mmol/l). For infants who are at risk of hypoglycemia because of alterations in maternal metabolism, intrinsic neonatal problems, or endocrine or metabolic disturbances, glucose monitoring should begin as soon after birth as possible. For those with values less than 36 mg/dl (2.0 mmol/l), close surveillance should be maintained and intervention recommended if concentrations remain low, regardless of the presence or absence of symptoms. In those infants in whom very low concentrations are detected (< 20–25 mg/dl; 1.1–1.4 mmol/l), therapeutic intervention should be initiated immediately. Newborn infants that are being fed by continuous parenteral nutrition will have persistently high insulin levels. As a result, their ability to manifest significant ketogenesis and the means by which to utilize alternate substrates will be impaired. Under these circumstances, maintainance of blood glucose concentrations in the higher therapeutic ranges (> 45 mg/dl; 2.5 mmol/l) is recommended. Similar recommendations might be made for infants with conditions such as hypoxia-ischemia, sepsis, or seizures in whom the demand for substrate to meet increased metabolic needs may be greater than supply (Cornblath, Hawdon et al. 2000; Yager 2002).

Incidence

There are conflicting reports about the incidence of hypoglycemia since it depends on the definition used, the methods by which blood glucose concentrations are measured, and those variables described above. In a study by Sexson et al (Sexson 1984) they found that 8.1% of 232 infants had glucose values of less than 30 mg/dl (1.6mmol/l) in the first hours of life and 20.6% had glucose concentrations less than 40 mg/dl (2.2 mmol/l). Also, Lubchenco et al (Lubchenco and Bard 1971) studied the incidence of hypoglycemia according to gestational age and weight. The 374 infants in whom blood glucose was obtained before their first feeding, the overall incidence of hypoglycemia was 32% for small for gestational age (SGA) infants, and 10% and 11.5% for appropriate for gestational age (AGA) and large for gestational age (LGA) infants, respectively, when less than 1.6 mmol/l was used as a definition. When less than 1.1 mmol/l was used as a defining level, the incidence decreased (Table 2). The overall incidence of neonatal hypoglycemia has been estimated at 1 to 5 per 1000 live births, but it is higher in at-risk newborns (30%). Studies have reported that 15 % of preterm and IUGR infants and 8% of LGA infants of diabetic mothers have hypoglycemia (McGowan 1999). Hence, irrespective of definition, hypoglycemia continues to be a common occurrence among the newborn population.

Table 2. Incidence of Hypoglycemia Classified by Birth Weight and Gestational Age
(Lubchenco and Bard 1971)

Birth Weight	Gestational Age	Blood Glucose < 1.6 mmol/l %	Blood Glucose < 1.1 mmol/l %
SGA			
	Preterm	67	40
	Term	25	21
	Post-term	18	9
AGA			
	Preterm	15	3
	Term	10	2
	Post-term	5	0
LGA			
	Preterm	38	13
	Term	4	2
	Post-term	7	0

Glucose Metabolism

Glucose is the primary fuel for energy production in the fetal and newborn brain and is essential for normal development to proceed. Animal and human studies indicate that brain glucose utilization is initially low and rises with maturation and increasing regional heterogeneity. With advancing age and functional activity, brain energy demands increase, and therefore brain glucose utilization is also increased (Vannucci and Vannucci 2000).

There is a linear relationship between the concentration of glucose in the mother and that of the fetus (Rosenblatt and Wolfe 1988). The fetus is entirely dependent on the mother for the continuous placental transfer of glucose and other nutritional requirements. The fetus is also capable of

using other alternate substrates such as amino acids and lactate. Glucose is stored in the liver and muscles in the form of glycogen and is released into the blood when cells need it for energy. In the fetus, glycogen storage does not occur before the 27th week (Mena, Llanos et al. 2001). Glycogen synthesis is initiated during the 2nd trimester of gestation and rises slowly until 36 weeks. Then glycogen content in the liver increases rapidly until full term. Gluconeogenesis is inhibited by high insulin levels in the fetus and is not functional in utero. Hepatic fatty acid synthesis and glucose uptake in adipose tissue are enhanced by insulin leading to triglyceride synthesis. This allows fat storage in adipose tissue during 3rd trimester. Glycogen and fat constitute stores available for metabolic changes at birth (Mitanchez 2007).

Maternal glucose supply is abruptly terminated (by umbilical cord section) at birth. For rapid adaptation, an endocrine stress response involving insulin and glucagon drives hepatic glycogenolysis, lipolysis, gluconeogenesis and fatty acid oxidation that generates lactate and ketone bodies as alternative fuels to maintain brain energy metabolism (Mitanchez 2007). Rapid hormonal fluctuations cause glycogen stores to be broken down to maintain the newborn's nutritional support. Glucose-6-phosphatase is an enzyme in the glycogenolysis and gluconeogenesis pathways that dephosphorylates glucose-6-phosphate so that the free glucose can be exported from the cell when needed. This enzyme, which is expressed at low levels in the newborn, rises within the first few days of

life (Burchell, Gibb et al. 1990). Estimated rates of glucose metabolism in the 1-day-old newborn are threefold greater than older newborns and infants (Bier, Leake et al. 1977). Nonetheless, glucose oxidation measurements in newborn infants indicate that only about 70% of cerebral energy requirements are met by glucose. Thus, the newborn brain is adapted to utilize ketone bodies and lactate to maintain cerebral energy levels. In the newborn brain, ketone bodies can be up to 40-fold greater than the adult brain, and lactate contributes remarkably in the first few hours of life.

In preterm infants, there is a larger drop in blood glucose level within the first few hours after birth. Since glycogen storage initiates during the third trimester, hepatic glycogen stores are limited in infants born before 28 weeks of gestation. Gluconeogenesis is then the major method of glucose production in these premature neonates. Some studies have shown that preterm infants have limited gluconeogenic ability due to immaturity of enzymes involved (Hume and Burchell 1993; Mitanchez 2007). Absence of stored glycogen and the time required to induce the enzymes required for gluconeogenesis in the immature, make hypoglycemia nearly inevitable in the first hours after birth if exogenous glucose is not administered.

Alternate Substrates to Glucose

Glucose oxidation can only support 70% of the brain energy requirements in term neonates (Mitanchez 2007). When there are situations in which glucose availability is limited, cerebral cells need to obtain their required energy through other substrates. Under these conditions, the perinatal brain is able to use and metabolize alternate substrates such as ketone bodies (acetoacetate and Beta-hydroxybutyrate that are produced during the metabolism of fats and synthesized in the liver during starvation for use as an alternative energy source to glucose), and lactate acid (Yager 2002; Mitanchez 2007). Although long chain fatty acid oxidation and ketone body production are low in the fetal liver, findings indicate an enhanced capacity for the brain extraction of ketone bodies from blood in newborns (Bougneres, Lemmel et al. 1986; Hawdon, Ward Platt et al. 1992). Hepatic ketogenesis is limited within the first hours following birth and significantly rises during the first 24 h after birth. During the 2nd and 3rd days following birth they exhibit high concentrations of ketone body. For the premature brain, ketogenesis is severely limited and fat stores are very low. In addition to low blood glucose levels during the first week after birth, they have low ketone body and free fatty acid concentrations, probably related to a combined failure of lipolysis and ketogenesis during this period (Hawdon, Ward Platt et al. 1992). Studies show the contribution of ketone body utilization to be 20–35% of brain energy metabolism in suckling newborn rat pups (Cremer 1982; Nehlig

and Pereira de Vasconcelos 1993; Nehlig 1996). As rat pup grows, ketone body utilization increases and peaks at PD14, and then diminishes to PD21 when cerebral glucose utilization is increasing and glucose becomes the predominant substrate for energy metabolism (Yager 2002). These findings coincided with the capacity of the immature blood-brain-barrier to transport ketone bodies at a rate that is threefold greater than glucose transport (DeVivo, Leckie et al. 1973; Yager 2002).

Under pathophysiologic conditions such as hypoglycemia, lactic acid appears to be the primary source of energy for the cerebral cells. In a study by Hernandez et al., 95% of brain energy requirements in normoglycemic newborn dogs were met by glucose while lactate and ketone bodies contributed 4% and 1%, respectively (Hernandez, Vannucci et al. 1980). With induction of insulin-induced hypoglycemia, and a concurrent decrease in cerebral glucose utilization, the share of lactate for brain oxidative metabolism became 58%. Vannucci et al. showed that, under these circumstances, there was no drop in cerebral high-energy phosphate levels owing to utilization of the alternate substrates in the lack of glucose (Vannucci and Vannucci 1978).

Cerebral Blood Flow, Glucose Utilization, and Brain Energy Metabolism

Animal and human newborn studies have indicated an inverse, linear relationship between blood glucose concentrations and cerebral blood flow (CBF) (Anwar and Vannucci 1988; Pryds, Greisen et al. 1988).

This increase in CBF was more significant in brain stem structures in studies in newborn dogs (Anwar and Vannucci 1988). These findings are consistent with other findings obtained from regional brain glucose utilization measurements during hypoglycemia that showed an association between low blood glucose and increased regional cerebral blood flow (rCBF) in all brain regions, ranging from 172% in parietal white matter to 249% in thalamus. Although in these studies regional cerebral glucose utilization (rCGU) was unchanged in most structures of the brain, there was a considerable drop in the occipital white matter structures and cerebellum (Mujsce, Christensen et al. 1989). It was also found that although glucose transport into the brain is enhanced during hypoglycemia by the increases in CBF, glucose delivery contributed less than 10% to the maintenance of CGU. Other studies in the newborn dog brain showed that in severe hypoglycemia (<1 mmol/l), cerebral metabolic rate for oxygen (CMRO2) was preserved in spite of a 50% reduction in CMRglucose. At this level, lactate became the dominant fuel for oxidative metabolism and its cerebral metabolic rates increased 10 times (Hernandez, Vannucci et al. 1980; Yager 2002). Similar studies in newborn dogs showed preservation of high-energy phosphate reserves during hypoglycemia (Vannucci, Nardis et al. 1981). It seems, therefore, that during neonatal hypoglycemia, CBF autoregulation is lost and that rather than glucose delivery, low energy demands maintain glucose homeostasis. In severe cases of hypoglycemia (<1 mmol/l), alternate

substrates, particularly lactate, appear to be the main substrate for cerebral energy requirements (Yager 2002).

Hypoglycemia and Hypoxia-Ischemia / Anoxia

Even though the immature brain appears to be resistant to isolated hypoglycemia, neonatal hypoglycemia is detrimental when superimposed on hypoxia-ischemia. Vannucci and Vannucci subjected newborn rat pups to anoxia in 100% nitrogen (Vannucci and Vannucci 1978). The experimental group was rendered hypoglycemic to 0.75 mmol/l (14 mg/dl) by intra-peritoneal insulin injection. Normoglycemic animals survived 10X as long as those that were hypoglycemic. In newborn dogs made hypoglycemic in combination with asphyxia, brain ATP concentrations fell by 61%, compared to those puppies with hypoglycemia alone, in whom ATP was preserved.

To determine the combined effects of substrate utilization and hypoxia-ischemia in the neonate, Yager et al subjected 7-day rat pups to hypoglycemia by either fasting them for 12 hours, or by subcutaneous injection of insulin (Vannucci and Yager 1992; Yager, Heitjan et al. 1992). Both control and experimental rat pups underwent hypoxia-ischemia by exposure to 8% oxygen combined with unilateral common carotid artery ligation. Although hypoglycemia was only mild in nature; 5.4, 4.3 and 3.4 mmol/l for control, insulin and fasted groups respectively, brain damage was significantly greater in the insulin treated animals than either of the other 2 groups. Fasted animals had the least damage, presumably due to

the enhanced ketogenesis and alternative substrate utilization displayed by this group.

In the human newborn, several studies have reviewed the effects of compounding hypoglycemia and perinatal asphyxia. Salhab et al (Salhab, Wyckoff et al. 2004) retrospectively reviewed 185 term infants with perinatal asphyxia defined by a cord pH of < 7.00. Fifteen percent of the infants had an initial blood sugar of < 40 mg/dl (2.2 mmol/l). These authors found a significant contribution of hypoglycemia to abnormal outcome, compared to those infants with blood sugars > 40 mg/dl. The authors did not comment in this paper about the duration of hypoglycemia. Additional complicating features were also present.

Hypoglycemia and Seizures

Young et al. compared depletion of cerebral high-energy phosphate stores in newborn puppies in two conditions; seizures alone, or in combination with hypoglycemia. Their ^{31}P NMR spectroscopy studies showed a marked reduction in phosphate stores in the latter (Young, Cowan et al. 1987). Seizures are associated with a rise in energy demand and subsequent increase in glucose utilization. When seizures are associated with hypoglycemia, supplies of glucose are further depleted and this puts the brain in a very vulnerable position (Yager 2002).

Etiology and Risk Factors

There are a variety of conditions and disorders that might cause hypoglycemia in the neonatal period. Prematurity and intrauterine growth

retardation (IUGR) are associated with decreased carbohydrate stores and might result in hypoglycemia. Infants of diabetic mother (IDM), are also at risk for hypoglycemia because maternal hyperglycemia leads to fetal hyperglycemia and hyperinsulinism followed by hypoglycemia in the newborn infant (Rozance and Hay 2006).

Sepsis, inborn errors of metabolism such as fatty acid oxidation defects and galactosemia can also result in neonatal hypoglycemia in newborn infants (Rozance and Hay 2006). Endocrine disorders such as hyperinsulinism due to islet cell hyperplasia, panhypopituitarism, and suppression of hypothalamic-pituitary-adrenal axis (HPA axis) are other conditions that might contribute to neonatal hypoglycemia. Likewise, asphyxiated infants, newborns with respiratory distress syndrome (RDS), small and large for gestational age (SGA and LGA) infants are at risk for low blood glucose concentrations (Yager 2002). Findings have indicated other significant risk factors for neonatal hypoglycemic brain injury include Low birth weight (<2.5 kg), history of poor feeding in the newborn period and lower segment cesarean section delivery (Udani, Munot et al. 2009).

Clinical Features and Symptoms

Although neonatal hypoglycemia can be asymptomatic, common clinical features which are associated with hypoglycemic encephalopathy include alteration in the level of consciousness, lethargy, somnolescence, irritability or stupor. Symptoms may also include floppiness, hypotonia, listlessness, hyper-reflexia, abnormal eye movements, jitteriness, tremors,

pallor, cyanosis, apnea, tachypnea, high-pitched cry, poor feeding, refusal to suck, sweating, hypothermia, temperature instability, drowsiness, seizures and coma (Volpe 2001; Yager 2002). Unfortunately, these clinical manifestations are subtle and are not necessarily specific to hypoglycemia and may be attributable to other pathologic conditions which almost always combine with hypoglycemia.

Hypoglycemia and Brain Damage

As hypoglycemia is often associated with other underlying compromises, evidence of pathologic injury to the brain as a result of pure neonatal hypoglycemia has been particularly difficult to obtain. When a neurological impairment is identified in a child who had diagnosis of hypoglycemia in the neonatal period, questions arise whether this disability is due to hypoglycemia per se or as a result of other factors or a combination (Williams 2005). In this regard, in vitro cell culture studies, as well as animal models of pure hypoglycemia have been helpful in documenting the effects of significantly low blood glucose on brain pathology. The effect of pure hypoglycemia on the developing juvenile brain has been documented in animal models including primates (Brierley, Brown et al. 1971; Volpe 2001). These studies have found that cerebral injury occurs as a result of prolonged and severe hypoglycemia, rather than mild or short-term hypoglycemia. The parieto-occipital cortex, hippocampus, caudate and white matter were most sensitive to prolonged hypoglycemia. And while mild hypoglycemia or mild HI did not result in

brain damage, the combination did result in cerebral injury (Brierley, Brown et al. 1971; Volpe 2001). However, these studies were done on more mature, juvenile animals, and determined only the short term outcome, pathologically. Therefore, no previous studies have evaluated the long term histological or behavioral effects of isolated hypoglycemia on late-preterm or near-term newborn brain, as determined in the current study.

While most hypoglycemic newborns do not develop neurologic sequelae, a few will have some degree of neurological impairment. Neuroimaging, electroencephalographic, metabolic and histopathologic findings show that profound and recurrent episodes of hypoglycemia can cause brain damage in newborn infants (Alkalay, Sarnat et al. 2005). This may present clinically as reduced head circumference, psychomotor impairments (Duvanel, Fawer et al. 1999), motor deficit, and mental retardation (Pildes, Cornblath et al. 1974). Studies have indicated the greater importance of recurrent or long periods of hypoglycemia compared with severity. In a comparison between severity and recurrence of low blood glucose as a predictable factor for long-term effects of neonatal hypoglycemia, Duvanel et al. showed that newborns with recurrent mild hypoglycemia had lower neuro-developmental scores than those with a single episode of severe hypoglycemia (Duvanel, Fawer et al. 1999). Symptomatic neonatal hypoglycemia may be associated with later neurodevelopmental impairement such as poor cognition and seizures.

Burns et al. showed neuroimaging findings and neurodevelopmental outcomes in symptomatic hypoglycemic term newborns. They found that white matter injury was not confined to the posterior regions and that hemorrhage, middle cerebral artery infarction, basal ganglia/thalamic abnormalities and cortical involvement were common. They also found that early MRI findings were more instructive than the severity or duration of hypoglycemia for predicting neurodevelopmental outcomes. Neurodevelopmental impairments seen at 18 months were related to the severity of white matter injury and involvement of the posterior limb of the internal capsule in their study. (Burns, Rutherford et al. 2008). Udani et al. 2009 found that microcephaly, severe mental retardation, autism, apraxia of hand use and cortical visual impairments are frequently observed in children with hypoglycemia (Udani, Munot et al. 2009).

Neuropathologic Features

In vitro NMR studies on energy metabolism of neurons and astroglia under various pathological conditions has shown that hypoglycemia *per se* did not significantly alter the high-energy reserves of either neurons or glia, even at levels as low as 0.1 mmol/l (Alves, Fonseca et al. 2000). In a study, Hertz et al. 1995 provided a substrate free medium without glucose and amino acids which does not allow for the utilization of any alternate substrate. When exposing immature astrocytes in culture with this medium, the immature cells were able to survive for almost twice as long as the mature astrocytes. This study obviously

points out the resistance of immature cells to substrate deprivation even though it is not necessarily an indication of the effects of hypoglycemia (Hertz, Yager et al. 1995). This may imply that newborn brains are more resistant to hypoglycemia.

Brierly et al (Brierly 1971) investigated the effects of insulin-induced hypoglycemia in a group of adolescent primates (physiologic parameters were controlled). Six of the 10 animals whose blood sugar was lowered to < 20 mg/dl (1.1 mmol/l) for 2 hours or more displayed selective neuronal necrosis throughout the cerebral cortices, with particular vulnerability in the parieto-occipital region, hippocampus, caudate and putamen. Similar findings were present in primates exposed to severe (<20 mg/dl) and prolonged hypoglycemia of > 6 hours. In these animals neuropathologic alterations primarily occurred in the basal ganglia, cerebral cortex, and the hippocampus (Myers 1971).

In the adult rat, Auer (Auer, Wieloch et al. 1984; Auer, Kalimo et al. 1985; Auer, Kalimo et al. 1985; Auer, Kalimo et al. 1985; Auer and Siesjo 1988; Auer and Siesjo 1993) and his colleagues have done extensive work defining the neuropathological consequences of severe hypoglycemia. In their series of papers, this group defined the timing, evolution and distribution of hypoglycemic brain damage in the rat. These investigators defined severe hypoglycemia and that level of blood glucose which caused electrocerebral silence (Auer, Olsson et al. 1984). Their previous studies had shown that glucose concentrations under these

circumstances were between 0.12 and 1.36 mmol/l. Over the course of these investigations, they described several important features of hypoglycemic brain damage that distinguishes it from ischemic injury. These include: 1) that infarction of brain tissue does not occur with hypoglycemia, 2) a superficial to deep gradient in the density of neuronal necrosis is seen in the cerebral cortex, 3) the caudatoputamen is involved more heavily near the white matter, and near the angle of the lateral ventricle, and 4) the hippocampus shows dense neuronal necrosis at the crest of the dentate gyrus (which is always spared in ischemia), and a gradient of increasing damage in the medial aspect of CA_1 (Auer, Kalimo et al. 1985; Auer, Kalimo et al. 1985; Kalimo, Auer et al. 1985). White matter injury was not particularly dealt with in these studies.

Unfortunately, in the human neonate, there is clearly a paucity of neuropathologic papers that provide insight into the contribution and distribution of injury in the newborn brain as a result of hypoglycemia. Anderson et al (Anderson, Milner et al. 1967) described 6 neonates who had been diagnosed with hypoglycemia and died within the first year of life for other non-CNS related causes. Blood glucose concentrations were <20mg/dl in all cases. In 4/6 neonates, the duration of hypoglycemia was greater than 36 hours. All infants were symptomatic. Histopathologically, they observed widespread necrosis of neuronal and glial cells in the cerebral cortex, hippocampus and basal ganglia. Within the cortex, the authors commented on a greater degree of involvement in the occipital

region than the frontal region. They also noted no predilection for the boundary zones between major blood vessels which often distinguishes ischemic lesions. Larroche et al. emphasized the white matter damaging potential of hypoglycemia, and demonstrated prominent periventricular leukomalacia in her series of newborns expiring of hypoglycemia (Larroche 1977).

Neuroimaging Abnormalities

Specific patterns of abnormality on CT and MRI scans of infants with diagnosed hypoglycemia have been a recent finding in the literature. Neuroimaging features of newborns who suffered from hypoglycemic-induced cerebral injury have been shown to have similar patterns. These findings have confirmed the vulnerability of occipital region to neonatal hypoglycemia (Barkovich, Ali et al. 1998; Filan, Inder et al. 2006; Yalnizoglu, Haliloglu et al. 2007). Spar et al. (Spar, Lewine et al. 1994) described a newborn with well documented hypoglycemia for at least 15 hours. The MRI scan demonstrated a predominance of tissue loss in the parenchyma of the occipital lobes, bilaterally. Barkovich et al. (Barkovich, Ali et al. 1998) described his group of 5 patients who suffered from hypoglycemia in the newborn period and emphasized the findings of white matter damage in the parietal and occipital lobes. Globus pallidus injury was present in only one of the infants described. Kinnala et al. (Kinnala, Rikalainen et al. 1999) published the findings of 18 full term infants with blood sugars <45 mg/dl (2.5 mmol/l). All were symptomatic. Only 3 of the

infants reported had hypoglycemia for greater than 24 hours with the longest duration being 33 hours. The mean blood sugar value was 25 mg/dl (1.4 mmol/l), with only 2 of the infants displaying sugars below 5 mg/dl (0.3 mmol/l). Four of the infants showed hyperintensity lesions on T_1 weighted images either in the occipital periventricular regions, or the thalamus. Ninety-four percent of the infants were developmentally normal at follow-up. Murakami et al. (Murakami, Yamashita et al. 1999) confirmed these latter results in his retrospective review of brain MRI in 8 term infants, all of whom were symptomatic and had blood glucose concentrations below 20 mg/dl (1.1 mmol/l). Once again abnormalities were consistently found in the parieto-occipital white matter in all but one child.

Within the last decade, a pattern of predominantly parieto-occipital white matter abnormalities, often in association with abnormal signal in the deep grey matter structures of the thalamus and/or basal ganglia has been identified on follow-up of those neonates who had experienced symptomatic hypoglycemia. Though the number of studies and patients is few overall, the abnormalities appear to be relatively distinct. Burns et al. found the patterns of cerebral damage associated with symptomatic neonatal hypoglycemia were more varied than described in previous studies. In their findings of neonatal brain injury, white matter involvement was not confined to the posterior regions, and cortical involvement, hemorrhage, basal ganglia and thalamic abnormalities, middle cerebral

artery infarction were seen. These authors also showed that in order to predict neuro-developmental outcomes in symptomatic hypoglycemic newborns, early MRI findings were more instructive than duration and severity of hypoglycemia (Burns, Rutherford et al. 2008). Alkalay (Alkalay, Flores-Sarnat et al. 2005) reported a case of term hypoglycemia and reviewed the available reports of associated imaging findings. Blood sugar findings were always < 25 mg/dl (1.4 mmol/l); they were generally low for a prolonged period of time, and; patients were symptomatic. When neuro-imaging was done in this group, it was abnormal, with over 80% showing consistent abnormalities of the occipital lobes.

Clinical and Neuro-developmental Outcome

Burns et al. showed neuro-developmental outcomes after symptomatic neonatal hypoglycemia until the age of 2 years old. Motor disabilities such as cerebral palsy (CP), abnormal cognitive abilities, speech and language delays, seizures before the age of 2 years, suboptimal head growth and vision abnormalities were some of the outcomes found in their study (Burns, Rutherford et al. 2008). Brand et al. studied the effect of neonatal hypoglycemia on neurodevelopment in healthy, large for gestational age (LGA), term infants. They found that transient mild hypoglycemia in healthy, term LGA newborns does not appear to be harmful to psychomotor development at the age of 4 years (Brand, Molenaar et al. 2005). Yalnizoglu et al. studied long-term prognosis of neonatal hypoglycemia in patients with MRI pattern of

damage typical for neonatal hypoglycemia who had prenatal and/or perinatal problems including IUGR, hypoxia-ischemia, indirect hyperbilirubinemia and sepsis. The neurologic sequelae of hypoglycemia in these children were developmental delays, learning and behavior difficulties, attention deficit hyperactivity disorder (ADHD), microcephaly, autism and cortical blindness (Yalnizoglu, Haliloglu et al. 2007). Duvanel et al. found a strong correlation between recurrent episodes of hypoglycemia and persistent neurodevelopmental and physical growth deficits until 5 years of age in small-for-gestational-age (SGA) infants. They suggested that recurrent hypoglycemia is a more predictable factor for long-term effects than the severity of a single hypoglycemic episode (Duvanel, Fawer et al. 1999). Lucas et al. studied the neurologic outcome of 661 preterm infants who weighed less than 1850 grams as a function of duration of hypoglycemia (table.3). They found an inverse correlation between number of days that glucose level were <2.6 mmol/l and mental and motor developmental scores. The relative risk of neurodevelopmental impairement in hypoglycemic infants with blood glucose <2.6 mmol/l for >5 days was 3.5 times greater than those without hypoglycemia (Lucas, Morley et al. 1988; Yager 2002).

Table 3. Neurodevelopmental Outcome of Infants Less than 1850 grams as a Function of Duration of Hypoglycemia (<2.6 mmol/l)

	Days of Hypoglycemia			
	0	1-2	3-4	>5
Number of Infants	29/177	44/284	11/51	13/31
Occurrence	16%	16%	22%	42%
Relative Risk		1.1:1	2.2:1	3.5:1

(Lucas, Morley et al. 1988; Yager 2002)

Hypothesis

Though reports of hypoglycemic brain injury focus on the parieto-occipital region as an area of interest, little mention is made of the confounding variables present in these infants, again placing into question the specific nature of the contribution of hypoglycemia to brain injury. In other words, this pattern of abnormality may represent a mixture of newborns exhibiting pure hypoglycemia or hypoglycemia in combination with other perturbations such as hypoxia-ischemia, sepsis, respiratory distress, etc.

Given the above discussion regarding the incidence, definition and compounding variables that influence the effects of hypoglycemia on brain injury and the neurodevelopmental outcome of the newborn infant, the direct effects of pure hypoglycemia on the immature brain remain unclear. The purpose of this research is therefore to isolate hypoglycemia as a variable and thereby isolating its effects on the newborn brain. The **specific aim** of this thesis is to determine the neuropathologic and behavioral effects of hypoglycemia *per se* on the immature rat brain and the importance of its duration and/or severity on brain damage.

We **Hypothesize** that:

Hypoglycemia, *per se*, in the absence of other metabolic and vascular perturbations, will *not* cause brain damage in the immature animal.

Research Methodology and Experimental Design

In order to test our hypothesis we developed a model of isolated hypoglycemia in immature rat pups. We determined outcome by assessing the following parameters: 1) Behavioral Outcome, 2) Histological and immunohistochemistry consequences on the immature rat brain, and 3) Alterations of excitatory amino acids and reactive oxygen species in the immature brains.

Animals

All animal testing has been in compliance with bioethics guidelines approved by the University of Alberta Animal Welfare and Policy Committee. Long-Evans male and female adult rats were purchased from Charles Rivers Laboratories (Montreal, PQ), housed in the Health Sciences Laboratory Animal facility (HSLAS) at the University of Alberta, and maintained on a 12-hour light/dark schedule. They received food and water *ad libitum* throughout the study. The animals were locally bred by our research associate in the Laboratory for Perinatal Brain Damage. Rat pups were delivered vaginally. Date of birth was considered to be post-natal day 1 (PD1), and litters are routinely culled to 10 pups. Within 48 hours of birth newborn rat pups were counted, weighed, sexed and returned to their dam. The pups were reared with their dams in conventional housing throughout the study. Rat pups were weighed on PD7 (late preterm or near term), PD15 and PD21 (2 year-old human newborn) (Andrews and Fitzgerald 1997; Hagberg, Ichord et al. 2002). On

PD7, pup weights determined exclusion criteria. Only those pups weighing 12 to 16 (14.6 ± 0.58) grams were utilized in the study. This age of brain development in the rat corresponds to 32-36 weeks of gestation in humans (late preterm infant) (Hagberg, Ichord et al. 2002).

Two groups of animals were identified for study purposes. Group I – Sham operated pups receiving normal saline (NS) and glucose infusion, and; Group II – Experimental pups receiving insulin and glucose infusion. Two rat pups also were maintained with their Dam to maintain lactation. The experimental paradigm is outlined in Figure 1 below.

Induction of Hypoglycemia

Experimental Paradigm and Preliminary Studies

In order to induce and maintain mild to severe hypoglycemia (blood glucose < 2.6 mmol/L) in the treatment group, and euglycemia in the Sham group, we developed the following model in our rat pups. All rat pups were initially removed from their dams and fasted for 2 hours. Subsequently, a PE-10 TYGON® flexible plastic tubing was inserted subcutaneously (SC) between the scapula, and held in place with cyano-acrylate glue.

Experimental rat pups were injected with 2.5 U/kg bovine insulin (SIGMA) subcutaneously followed by the infusion of 1.25 u/kg/hr insulin in 10% glucose/normal saline, one hour afterwards between the scapula. The pups in the sham group received normal saline injections followed by

infusions of 15% glucose/NS. The rate of infusion for the insulin and normal saline was 25 µl/hr. All pups were placed in glass jars in a thermo-regulated incubator adjusted at 34-35°C (nesting temperature). In both groups, the infusions continued for 17-34 hours, in order to obtain the desired levels and duration of hypoglycemia. Surviving rat pups were then categorized retrospectively to Long-Mild, Long-Moderate, Long-Severe, Short-Mild, Short- Moderate, or Short-Severe groups and used for behavioral assessment and subsequent neuropathologic and neurochemical determination.

Glucose concentrations were measured throughout the period of infusion starting at 0 hour (before insult) and at either 1, 6 12, 18, 24, 30 and 32 hours during the procedure as well as two hours post-recovery, with no more than four samples per animal (Figure 1). For blood glucose measurements, tail snips were obtained, and small quantities of blood dabbed on to standard glucose strips, that were then measured using an ONETOUCH ULTRA2 glucometer (LifeScan, Inc.).

In order to obtain hypoglycemia with desired duration in our experimental animals and euglycemia in the control pups we initially followed the early findings obtained from a previous study (Yager, Heitjan et al. 1992). Further experiments were performed to determine the concentration and/or rate of injected/infused insulin and glucose required to obtain mild to severe hypoglycemia, with the end point being the degree of hypoglycemia obtained. We tried different pre-injection fasting times,

insulin/glucose concentration/infusion rate, and etc. in our preliminary work (Table 4) to achieve our goals.

Fasting time before Injection	0, 1, 2 (hour)
Injected Insulin Concentration	2.5 U/kg
Infusion Rate	1, 1.25, 1.5 U/25µl/kg/hr
Glucose Concentration in Experimental Pups	7.5%, 10%
Glucose Concentration in Control Pups	12.5%, 15%
Delay Between Injection and Infusion	1, 2, 3 (hour)

Table 4. Preliminary work performed to determine the intervals and concentration and/or rate of insulin/glucose injection and infusion required to maintain euglycemia and obtain desired hypoglycemia.

Following the induction of hypoglycemia, rat pups were allowed to recover with their dams for either 24 hour after the end of hypoglycemia or 21 post-natal days. At 24 hours following hypoglycemia rat pups were assessed for neuropathologic injury using Flouro-Jade B. Those recovered to 21 days of age were assessed for early reflex behaviors and open field test from PD9 to PD21, as described below. In addition, neuropathologic and immunohistochemical assessments were obtained for determination of neuronal injury, loss of tissue mass and neuronal degeneration (H&E, Neu-N), myelinogenesis (MBP), and the astrocytic response to injury (GFAP). In separate groups of animals, rat pups were

immediately euthanized at the end of the hypoglycemic period and their brains were regionalized and stored at -70 °C for later neurochemical evaluations (Excitatory amino acids and oxidative stress markers).

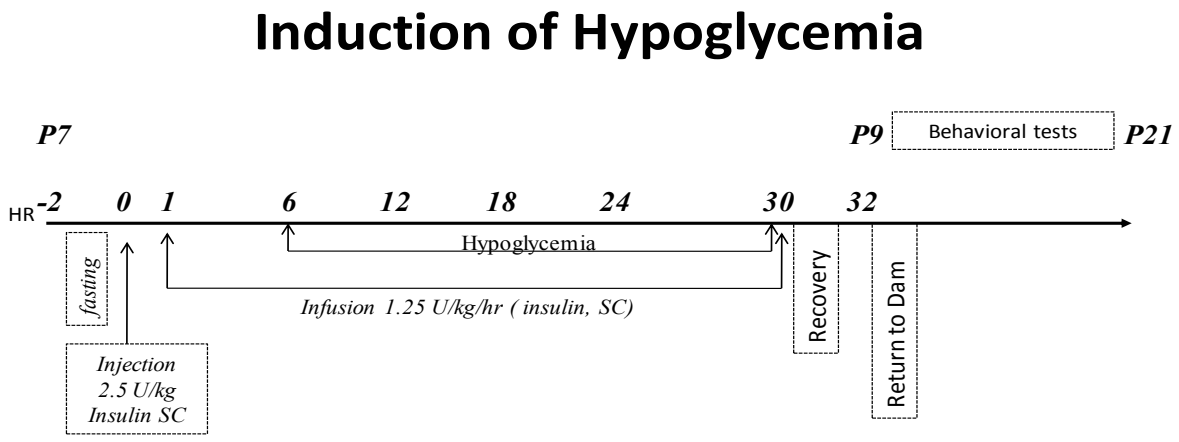


Figure 1. Method of induction of hypoglycemia in PD7 rat pups. This flow chart indicates the concentrations of insulin injection/infusion, intervals and time points at which blood glucose concentrations were measured as well as the behavioral tests that were performed during our experiments.

Hypoglycemic Criteria

In order to assess the effects of varying concentrations of hypoglycemia on the brain, we classified the blood glucose concentrations ranging from normal to severe (Table 5), based on those criteria determined in the literature. We also rendered rat pups hypoglycemic for different durations; short-term and long-term hypoglycemia (Table 6).

Hypoglycemia Criteria

Severity of hypoglycemia	Glucose level (mmol/L)
Normal	> 2.6
Mild	1.7 – 2.6
Moderate	1.2 – 1.6
Severe	≤ 1.1

Table 5. Hypoglycemia Severity Criteria

Duration of Hypoglycemia	Time (hour)
Short-term	< 12
Long-term	≥ 12

Table 6. Hypoglycemia Duration Criteria

Assessment of Neurobehavioral Development in Hypoglycemic Rat Pups

Neurological Signs/Reflexes and Behavioral Tests

Early reflex and behavioral tests (Fox 1965; Bekoff and Trainer 1979; Ba and Seri 1995; Gramsbergen and Mulder 1998; Keller, Saucier et al. 2004; Zhuravin, Dubrovskaya et al. 2004; Kiss, Tamas et al. 2005; Lubics, Reglodi et al. 2005) were performed to assess neurodevelopmental outcomes in the animals from PD9 to PD21. In order to do this, the rat pups were removed from their home cage for testing every afternoon. Newborn rat pups are extremely prone to hypothermia due to their immature thermoregulatory system (Kreider and Blumberg 2005). To maintain euthermia and avoid temperature-related behavioral alterations newborn rats were tested in an incubator maintained at 34.5ºC where possible or under a warming lamp (31ºC) for tests that could not be performed inside the incubator. Postnatal day of attaining each individual reflex (day of appearance) for each pup was recorded except for the righting and bar holding reflexes where the duration of time to accomplish the task, each day, was recorded. The following reflex tests were adapted from the original work of Fox and Lubics (Fox 1965; Lubics, Reglodi et al. 2005).

1- **Body Righting Reflex**

The pup is placed in the supine position and the time to turn over (with all four paws touching the surface) is recorded (principally labyrinthine

and body righting mechanisms). The animal should be able to complete this task in less than 5 seconds. This test was scored as 0 (≤2 sec), 1 (2-5 sec) and 2 (≥5 sec). This test was videotaped and time to perform body righting was recorded from PD 9 until either the time the pups performed the test within 5 seconds for two consecutive days or PD13.

2- **Fore/Hind-limb Grasping Reflex**

A small probe (metal wire) is placed in the centre of the "palm" of the fore or hind limb. The ability of the pup to grasp at the probe is recorded. The day each pup could grasp the instrument with both, fore or hind limbs, was the postnatal day of attainment for that reflex. A scoring system of 0 to 2 is used for this test. It is scored as 0 (no reflex), 1 (one limb reflex) or 2 (positive reflex in both hind limbs). This reflex was tested from PD9 until a score of 2 was attained for two successive days.

3- **Hind-limb Placing**

The animal is held suspended and the dorsal surface of the hind paw is rubbed against the edge of an object. The ability of the pup to lift its paw and place it on the top of the tabletop is measured. Beginning on PD9, the day each pup performed the placing task with both hind limbs was the day of attainment. Five trials are performed and scored as 0 (no reflex), 1 (one limb reflex) or 2 (positive reflex in both hind limbs).

When attaining a score of 2 for two successive days, the test was stopped.

4- **Gait**

Beginning on PD9 the rat pups were placed in middle of a 150 mm diameter white paper (circle) and the time for the two forelimbs to come out of the circle were recorded. The day they began to move off the circle with both forelimbs within 30s, was recorded. Longer than 30s to perform this task was considered a negative result. When capable of doing the test within 30 seconds for two consecutive days, the test was stopped. This test was scored as 0 (not able to do in 30 sec) or 1 (done within 30 sec).

5- **Normal posture**

This test is performed as a sign of maturation in which the posture of the limbs, particularly the hind limbs, was monitored. Rat pups crawl before PD10. Then they start using four limbs to maintain a quadruped stance. Beginning on PD14, the first day the pups could hold all four limbs beneath their body both during rest and locomotion is considered attainment of a mature posture (Bekoff and Trainer 1979). The end point was a positive test for two consecutive days.

6- **Bar holding** (motor coordination, strength test)

This test primarily measures strength; an important aspect of motor performance. To test changes in strength of the animals, rat pups

were suspended by their forepaws on a horizontal wire bar, stretched horizontally 40 cm over a foam pad. The time the pups fell off the bar was recorded using a stop watch. Rat pups should be able to do this at 12 days of age. This test was performed from PD12 to PD18.

7- **Auditory Startle**

This test is another sign of maturation. A loud sharp noise (bell or clapping hands) causes an immediate startle response, seen as a sudden extension of the head and fore and hind limbs which are then withdrawn and a crouching position is assumed, or there is flight (escape-avoidance) behavior at a certain age. Beginning on PD12, the first day of a startle response from a clapping sound was considered the day of attainment (Beard, Felt et al. 2006) and attaining the positive test for two successive days was the end point of the test. This test is scored as 0 (no reaction to startle) or 1 (positive reaction).

8- **Acceleration Righting**

When the rat is dropped with its back facing down it should be able to turn in mid air and land on its feet (statokinetic labyrinthine response). The rat was held back down and dropped from a height of 20 cm into foam or cotton. This test began on PD12 and was videotaped in order to view the landing of the animals in slow motion. The day in which the rat pup was able to turn and land on all fours was recorded. Best of two trials was considered as the result and was scored as a 3 point system 0 (no turning, lands on the back), 1 (partial turning, lands on

side) or 2 (complete turning, lands on four limbs). The day each pup scored a 2 was considered the day of attainment. The test was ended if positive for two consecutive days.

9- **Eye opening**

This test is also a sign of maturation. Beginning on PD14, the day that each eye opened was recorded (Beard, Felt et al. 2006). Even if the eye is half-opened, it is considered as positive. This test is scored as 0 (closed eyes), 1 (one eye opened) or 2 (both eyes opened). The first day in which both eyes were opened was considered the day of attainment and two successive days of positive test was the end point.

10- **Open Field Activity**

This test assesses general rat behaviors including locomotor activity, exploration cognition, excitability, emotionality, memory, anxiety and fear (Walsh and Cummins 1976). A quiet room with a constant temperature was used for this test. The home cage containing the animals was transferred into the testing room for acclimatization at least 1 h before the beginning of the experiment. The floor of a square Plexiglas board (45 cm× 45 cm) with Plexiglas sides (30 cm high) was divided into 16 squares (4×4 areas) (Figure 2). Every PD21 rat pup was placed into the central area facing the same direction. Whole 5 min exposure was recorded on video by a blind observer and evaluated off-line to observe various motor and exploratory behaviors. Four different behaviors were assessed within this time; ambulation,

rearing (Figure 3), head-lifting (Figure 4) and grooming (Figure 5) (Lubics, Reglodi et al. 2005; Kiss, Hauser et al. 2007). The animal's behavior in the open field chamber not only depends on the chamber itself but also on its surrounding environment and conditions. Illumination, noise and environmental odors affect the animal's behavior and consequently the results of the test. Findings have expressed high illumination as "stressful" index of emotionality. For instance, with the exception of Candland and Nagy (Candland and Nagy 1969), who reported the reverse, studies have shown diminished locomotor behavior in high levels of illumination. Valle (Valle 1970) indicated less ambulation and rearing under higher illumination (Walsh and Cummins 1976). Definitely, any abrupt loud noise can considerably inhibit locomotion and even induce prolonged immobility in a variety of species including rats (Hofer 1970; Walsh and Cummins 1976). White noise, however, might increase (Livesey and Egger 1970) or decrease (Bindra and Spinner 1958) locomotion and ambulation. Environmental odors also may affect the animal's behavior in the open field. The odor of the opposite sex (Satinder 1969) and the caretaker (Mccall, Lester et al. 1969), who has reared the animal, might cause the animal to spend more time on a specific side of the test field. Given the above, we cleaned the open field board using a glass cleaner (Windex) after testing each rat pup to eliminate the odors of the previous tested animals. All behavioral tests were performed

between 1 and 3 pm and in a consistent environment, inclusive of lighting, temperature, surroundings, and quietness as possible, and by the same experimenter. They were then scored by a blind observer.

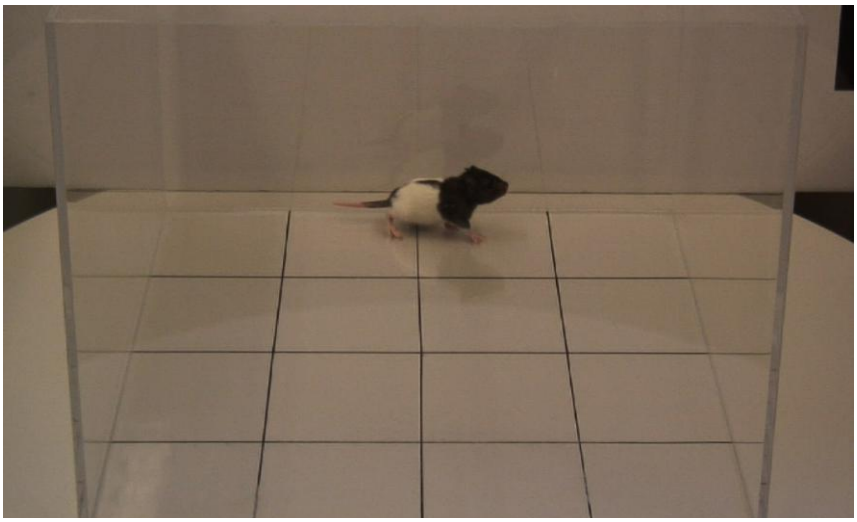


Figure 2. Open Field apparatus.

Figure 3. Open-Field, Rearing - an index of locomotor activity.

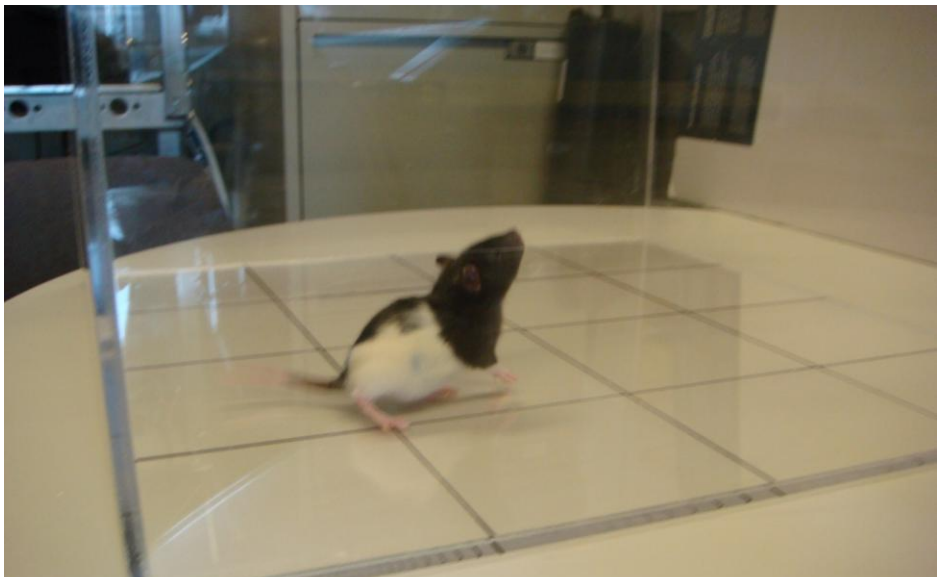


Figure 4. Open-Field, Head-Lifing - an index of exploration.

Figure 5. Open-Field, Grooming – negatively related to indexes of high-activity states.

Neuropathology and Immunohistochemistry

Animals were sacrificed at either P9 or P21 to determine the evolution of histopathologic alterations caused by the varying levels of hypoglycemia, and to ascertain whether early alterations are transient or permanent in nature. Animals were deeply anaesthetized with 5% Isoflurane USP in 30% oxygen balanced nitrogen, followed by immediate decapitation. The brains were quickly extracted and were fresh frozen in Isopentane. Brains were then stored at -70^{o}C until sectioning by cryostat (Leica, cryocut 1800).

Coronal brain sections (10 µm) were cut on a cryostat (-16 to -17 ˚C depending on the room's temperature and humidity) at the level of the anterior commissure (approximately 1.6 mm anterior to bregma),

mamillary bodies (1 mm posterior to bregma), and through the midbrain (2.5 mm posterior to bregma) (Sherwood and Timiras 1970). Sections were then mounted on slides and stored at -20 ˚C to be stained and used for immunohistochemistry and other assessments (described later). In order to assess the brain structural and histological changes in the rat pups such as neuronal death, inflammatory response, neuroglial changes and myelination; Neu-N, GFAP, Fluoro-Jade, and MBP were performed at either PD9 (FJB) or PD21 (Neu-N, GFAP, and MBP) (Figure 8).

In our experiment we assessed cerebral cortex, caudate, thalamus, CA1 of hippocampus considering their vulnerability to acute hypoglycemia in developing and mature rodents shown in previous studies (Auer, Wieloch et al. 1984; Kim, Yu et al. 2005; Yamada, Rensing et al. 2005) as well as hypothalamus owing to its important role in brain energy homeostasis and glucose sensing (Marty, Dallaporta et al. 2007). Corpus callosum, internal capsule and reticular nucleus of thalamus (TRN) were also assessed.

Fluoro-Jade B (FJB)

A useful stain for determining neuronal injury in developing and mature rodents is FJ-B (Tkacs, Pan et al. 2005; Yamada, Rensing et al. 2005; Suh, Hamby et al. 2007; Zhou, Qian et al. 2008) which is a reliable, sensitive, simple and fast high affinity fluorescent marker of degenerating neurons and their processes (Schmued, Albertson et al. 1997; Schmued and Hopkins 2000) and stains only dead or dying neurons in brain tissue.

It stains the cell bodies, dendrites, axons and axon terminals of degenerating neurons but does not stain healthy neurons, myelin, or vascular elements. Degenerating neurons stained with Fluoro-Jade appear bright green against a dark background (Schmued, Albertson et al. 1997). Since the 1-day post-insult time point represents the maximum FJB staining and the peak number of FJB-positive neurons are diminished after 46-96 post injury, we chose to stain our rat brains with FJ-B at the peak of 24 hours after the termination of hypoglycemia (Anderson, Miller et al. 2005).

Fluoro-Jade Staining Procedure. Brain sections were mounted onto Superfrost/Plus slides and air dried overnight. When fully dry, the slides were post-fixed in formalin and then immersed in 100% ethyl alcohol for 3 minutes followed by 1-minute in 70% alcohol and 1-minute in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate and gently shaken for 15 minutes. The slides were rinsed for 1 minute in distilled water and then transferred to the Fluoro-Jade staining solution in the dark room and stained for 30 minutes. A 0.01 % stock solution of the dye was prepared by dissolving 10 mg of Fluoro-Jade B (Chemicon) in 100 ml of distilled water. The 0.001 % working solution of Fluoro-Jade was prepared by adding 20 ml of the stock Fluoro-Jade solution to 180 ml of 0.1 % acetic acid in distilled water. After staining, the sections were rinsed 3 times for 1 minute each in distilled water. Excess water was drained off, and the slides were rapidly air dried on a slide

warmer at 37°C. When dry, the slides were immersed in xylene and then coverslipped with D.PX. (Aldrich Chemical Co, Milwaukee, WI), a nonaqueous and nonfluorescent plastic mounting medium and stored in the dark. Sections were examined with an epifluorescence microscope using a filter system suitable for visualizing fluorescein or fluorescein isothiocyanate (FITC) (eg, the Leica FITC filtercube B-2 has an excitation filter at 450-490 nm and a barrier filter at 520 nm). The resulting slides are very stable and require no special storage conditions or antifading agents (Schmued and Hopkins 2000).

Degenerating neurons were counted in FJB-stained sections (0.6 mm anterior and 3 mm posterior to bregma) (Sherwood and Timiras 1970) in cortex (anterior, posterior, and piriform), CA1 of hippocampus (medial and middle sectors), caudate, thalamus, TRN and hypothalamus at 400X magnification (area of 0.09 mm^2).

Immunohistochemistry

Glial Fibrillary Acidic Protein, Myelin Basic Protein, Neuronal Nuclei

Brain tissue sections were stained with glial fibrillary acidic protein (GFAP), to assess astrocytic infiltration and myelin basic protein (MBP) to examine development of myelinated white matter fibres and TRN optical density. Likewise, neuronal nuclei (Neu-N) was performed for detection of

loss of neurons and abnormal neuronal morphology in hypoglycemic rat brains.

Glial Fibrillary Acidic Protein (GFAP)

Glial fibrillary acidic protein is an intermediate filament protein that is found in glial cells such as astrocytes and is involved in the structure and function of the cell's cytoskeleton. GFAP helps to maintain astrocyte mechanical strength, as well as the shape of cells. GFAP is upregulated in response to injury to the central nervous system and is used as a marker for astroglial reactivity in the brain. In response to brain injury, these GFAP immunoreactive cells might show an activated astrocytic morphology with larger, swollen cell body as well as short and thick processes (Sizonenko, Camm et al. 2008).

Studies have demonstrated that radial glia cells are the predominant glial phenotype in the neonatal cortex (Rakic 2003; Sizonenko, Camm et al. 2008). In the rodent brain, the radial glia scaffolding progressively disappears during the first two postnatal weeks and is absent after P15 (Kalman and Ajtai 2001; Sizonenko, Camm et al. 2007; Sizonenko, Camm et al. 2008). Moreover, it is suggested that radial glia gives rise to GFAP-positive astrocytes therefore indicating a lineage relationship between these two cell types (Schmechel and Rakic 1979; Voigt 1989; Cameron and Rakic 1991; Sizonenko, Camm et al. 2008). Infiltration of activated astrocytes into an area of the brain indicates brain injury in that specific region of the brain. Since it takes some time for glial cells to infiltrate and

move towards the damaged area, GFAP immunohistochemical staining was performed on P21 animals.

GFAP Staining Procedure. Briefly, frozen sections were post-fixed in formalin and then dehydrated in a graded series of ethanol washes and cleared in xylene. Sections were then washed with 0.3% hydrogen peroxide to quench endogenous peroxidases and subsequently blocked with normal goat serum mixed with Triton X-100 (0.2%). Sections were then allowed to incubate with the primary antibody (Polyclonal Rabbit anti-GFAP, DakoCytomation, Glostrup, Denmark) overnight. After rinsing and 30 minutes of incubation with the secondary antibody (Goat anti-Rabbit IgG, Vector), the sections were rinsed again and incubated with an avidin-biotin complex (ABC, Vector Laboratories). The immunoreactivity is visualized with diaminobenzedine tetrahydrochloride (DAB, Sigma) (Hsu and Raine 1981).

In the brain sections stained with GFAP, in addition to assessing different areas of the brain looking for possible alterations and infiltration, medial and middle sectors of CA1 and two regions of cortex in both anterior and posterior sections were selected (1 mm anterior and 3 mm posterior to bregma) (Sherwood and Timiras 1970). Images were captured at 400X magnification (area of 0.091 mm^2) from the selected regions by using a microscope (Leica ATC 2000) and a Spot Flex Camera (Diagnostic Instruments, Sterling Heights MI) using Spot 4.5 software (Diagnostic Instruments, Sterling Heights MI). Then the GFAP

immunoreactive astrocytes with hypertrophic cell body and short thick processes, as described above, were marked using Adobe Photoshop CS2 software and counted.

Myelin Basic Protein (MBP)

White Matter Thickness and Densitometry

Myelin basic protein is a protein believed to be important in the process of myelination of nerves in the central nervous system (CNS) and MBP immunostaining can reveal white matter injury. Studies on HI-induced brain injury in rodents have indicated that quantitative measurement of MBP immunostaining provides a sensitive indicator of acute oligodendroglial injury in neonatal rodent brain. The loss of MBP immunostaining reflects oligodendroglial dysfunction, but not necessarily cell death. Mechanisms that could contribute to HI-induced loss of MBP immunostaining include reduced synthesis and increased degradation of MBP mRNA and protein. In fact, both death of oligodendrocyte progenitors and reduction of MBP mRNA and protein synthesis in surviving oligodendrocytes contribute to the loss of MBP immunostaining (Liu, Silverstein et al. 2002).

Myelination is not visible in sections of the corpus callosum of rat brain until the 11 - 12th postnatal days and as the animals grow myelination increases so that it becomes significant at PD30 (Kolb and Tees 1990). In the developing rat brain during the 2nd and 3rd postnatal

weeks, there is a substantial increase in MBP immunostaining (Biran, Joly et al. 2006). Assessment of maturational alterations in MBP immunostaining has revealed that on PD7, MBP immunostaining is barely detectable in rat brain. On PD12, however, it is readily identified, concentrated in white matter tracts of the external and internal capsules. In addition, at PD12, subtle periaxonal staining can be discerned within the striatum. On PD21, there is a further rise in MBP staining, but the widespread adult distribution has not yet obtained. At this age, there is more intense immunostaining, both within white matter tracts (the corpus callosum, the anterior commissure, the striatum) and also extending into adjacent gray matter cortical regions (Liu, Silverstein et al. 2002). Due to lack of myelination in younger animals, white matter integrity was evaluated on P21 rats by densitometric analysis of MBP (SMI 94; Cedarlane Laboratories Ltd., Ont., Canada) immunostaining (described below).

MBP Staining Procedure. In brief, frozen sections were post-fixed in methanol. Sections were then allowed to incubate with the primary antibody (monoclonal mouse anti-MBP, Covance) overnight and washed with 1% hydrogen peroxide to quench endogenous peroxidases and subsequently blocked with normal horse serum mixed with Triton X-100 (0.1%). After rinsing and 30 minutes of incubation with the secondary antibody (biotinylated horse anti-mouse IgG, Vector), the sections were rinsed again and incubated with an avidin-biotin complex (ABC, Vector

Laboratories Inc., Burlingame, CA, USA). The immunoreactivity is visualized with diaminobenzedine tetrahydrochloride (DAB, Vector Laboratories Inc.). (Hsu and Raine 1981).

Densitometric Analysis of P21 MBP-Stained Rat Brains

Ten micron sections were stained for MBP as previously described. Whole brain images of MBP-stained sections (1 mm anterior and 3 mm posterior to bregma) (Sherwood and Timiras 1970) were taken with a Spot Flex Camera attached to a Leica GZ6E stereoscope (Leica Microsystems, Richmond Hill ON, Canada) using Spot 4.5 software (Diagnostic Instruments, Sterling Heights MI). Densitometry was measured using Image J computer program ver 1.41 (reference - Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.) calibrated with a Kodak No. 3 Calibrated Step Tablet scanned with an Epson Expression 1680 Professional scanner. A 0.035 mm^2 area of the corpus collosum immediately beside the midline was measured in both anterior and posterior sections (Figure 6 a&b-1). In posterior sections, a 0.035 mm^2 area of the TRN was also measured from the most lateral edge, right beside the internal capsule, in one hemisphere of the brain (Figure 6-b-3). The same area of the dorsal cortex beside the midline, that did not show MBP reactivity, was also measured in anterior sections to determine background staining levels (Figure 6-a-2). In the posterior sections, CA1 of hippocampus was chosen as the area of the brain with no MBP

reactivity to determine background staining levels (Figure 6-b-2). This background level was then subtracted from the optical density of the corpus collosum and/or TRN, in the same section. This accounts for variability in staining results that occurs between different batches of immunohistochemistry.

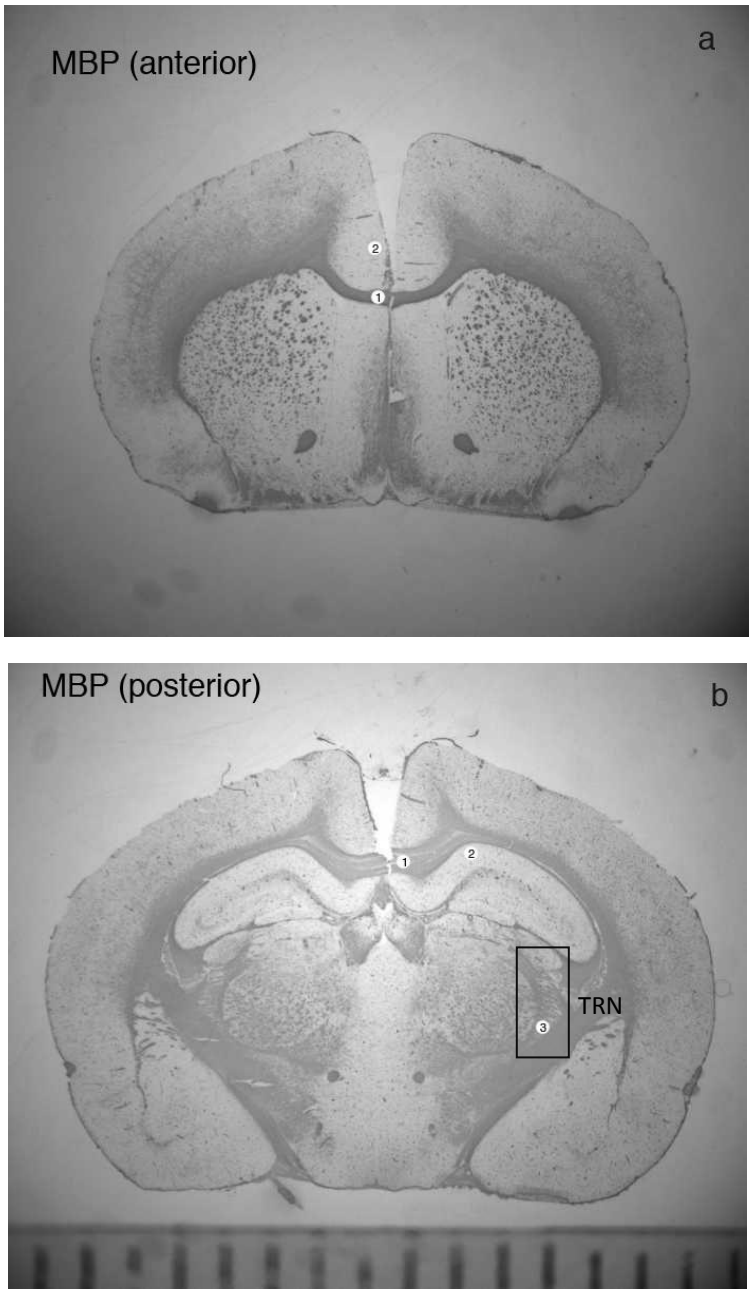


Figure 6. MBP-stained sections illustrating the regions where densitometric analysis were performed. a – Corpus callosum (1) at anterior sections and b – Corpus callosum (1) and TRN (3) at posterior sections.

Neuronal Nuclei (Neu-N)

This immunohistochemical staining is a marker of neurons and is used to detect loss of neurons and abnormal neuronal morphology in rat brain (Lin, Fan et al. 2009). To evaluate the effects of hypoglycemia on neuron loss and to determine if there is any mature lesion due to hypoglycemia, we detected Neu-N immunopositive cells by immunohistochemistry in hippocampus CA1 region as well as thalamic reticular nucleus (TRN) at P21. Less neuronal nuclei (Neu-N) positive neurons due to hypoglycemia in any region of brain could be indicative of neuron loss and brain damage in that area.

Neu-N Staining Procedure. Briefly, frozen sections were post-fixed in formalin and then cleared and dehydrated in a graded series of ethanol washes. Sections were then washed with 1% hydrogen peroxide to quench endogenous peroxidases and subsequently blocked with normal horse serum mixed with Triton X-100 (0.2%). Sections were then allowed to incubate with the primary antibody (monoclonal mouse anti-NeuN, Millipore) overnight. After rinsing and 30 minutes of incubation with the secondary antibody (biotinylated horse anti-mouse IgG, Vector), the sections were rinsed again and incubated with an avidin-biotin complex (ABC, Vector Laboratories). The immunoreactivity is visualized with diaminobenzedine tetrahydrochloride (DAB, Vector) (Xiong, Yang et al. 2009).

The count was determined by centering the microscope field of view (400X magnification) on medial, middle and lateral sectors of CA1 of hippocampus (Figure 7) (Colbourne and Corbett 1995) and the most lateral portion of the TRN (right beside the internal capsule). Images were captured from these views (area of 0.091 mm^2) (2.7 mm posterior to bregma) (Sherwood and Timiras 1970) and neuronal nuclei positive neurons were counted.

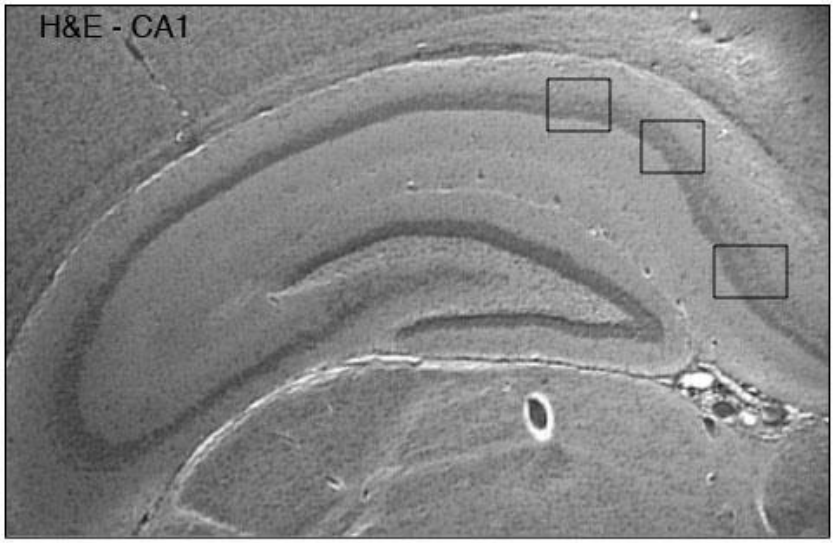


Figure 7. Medial, middle and lateral sectors of CA1 of hippocampus from which images were taken and were evaluated in our study.

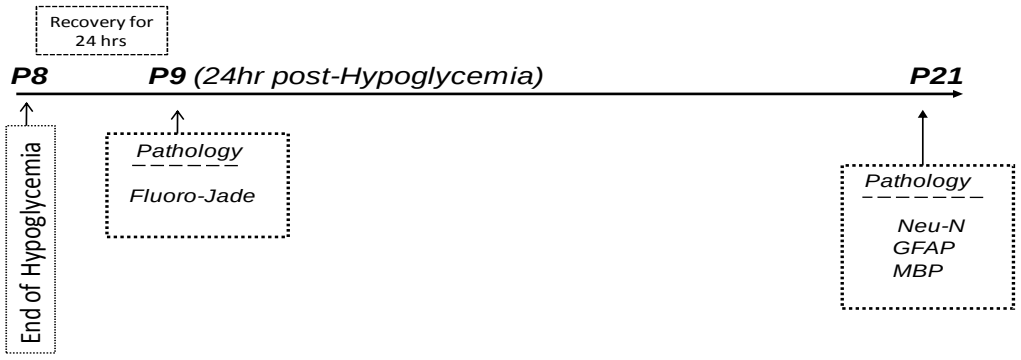


Figure 8. Postnatal days at which early and late pathologic studies were performed.

Measurement of Excitatory Amino Acids and Markers of Oxidative Stress (Matrix Metalloproteinase-2 and Redox Ratio)

To determine the effects of pure hypoglycemia on the level of excitatory amino acids and reactive oxygen species (forms of oxygen that have enhanced chemical reactivity compared with the normal oxygen molecule; many are free radicals) on the immature rat brain the pups were categorized as being sham or experimental. Hypoglycemia and euglycemia was maintained as described previously. Sham pups and the pups with 12-15 hours severe hypoglycemia (Blood Glucose ≤1.1 mmol/L) were immediately decapitated (Figure 9). The cortex, hippocampus and thalamus were rapidly removed and placed on dry ice. It took no longer than 2 minutes to remove the samples from the brain. All samples were stored at -70ºC until EAA/ROS analysis to ascertain the acute alterations

in the immature brain as a result of severe, prolonged hypoglycemia. The samples were sent out for determination of brain amino acids and MMPs. High Performance Liquid Chromatography (HPLC) analyses for the amino acids glutamate (Glu), γ-amino-butyric acid (GABA), glutamine (Gln), aspartate (Asp), L-serine, D-serine, glycine (Gly), arginine (Arg), taurine (Tau) and alanine (Ala) were performed by Dr. Kathryn Todd's laboratory. Procedures used were as per the methods of Jantzie and Todd (Jantzie, Rauw et al. 2006). MMP-2 zymography analyses and Redox Ratio, as an indicator of oxidative stress, were performed by Dr. Po-Yin Cheung's laboratory as per the methods of Schulz, Stevens and Cheung. The redox ratio was calculated as the ratio of reduced glutathione, determined by subtracting GSSG (oxidized glutathione) from GSH (total glutathione) levels, to GSSG (Redox Ratio = GSH – GSSG / GSSG) (Schulz, Sawicki et al. 2004; Richards, Todd et al. 2006; Johnson, Bigam et al. 2007; Stevens, Churchill et al. 2008).

Six sham and six long-severe hypoglycemic brains were analyzed in our study from which one hemisphere was used for EAA and the other one for ROS assessment randomly. The concentrations of EAA, MMP-2, and glutathione were then compared with those of sham rat pup brains.

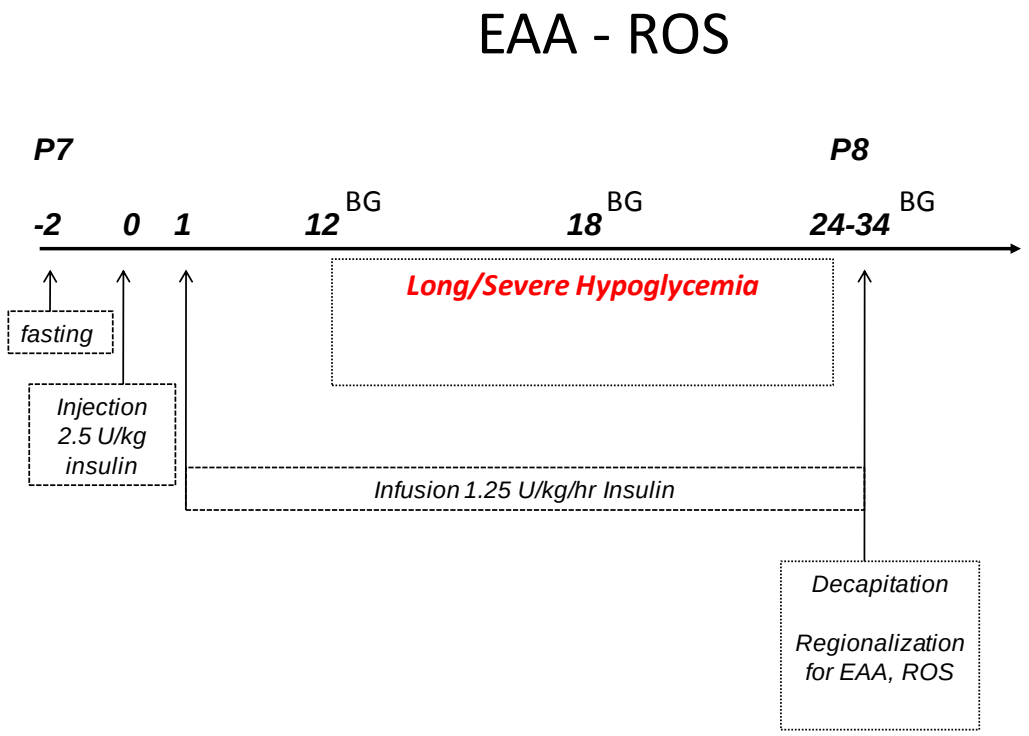


Figure 9. Methodology of hypoglycemia induction for EAA/MMP assessments.

Statistical Analyses

Comparisons were made using the student t-test and one-way Analysis of Variance (ANOVA). Tukey HSD or post hoc tests was used where necessary to determine inter-group differences. Differences in mortality rate were determined using the Fisher Exact Test. Data are presented as mean ± standard error of the mean (SEM). Significance was set at $p < 0.05$.

Results

Number of animals

We used a total of 328 rat pups in our experiments. Of these, 265 pups were induced with hypoglycemia of which 75 animals died during the course of hypoglycemia (Table 7). Therefore a total number of 190 hypoglycemic rat pups remained for our experiments; 49, 39, 26, in the long – mild, moderate, severe and 28, 24 and 24 in the short – mild, moderate, severe categories, respectively (Table 7). The other 63 pups were used as sham.

Table 7. Number of experimental animals by gender.

Numbers	Female	Male
Hypoglycemic (190)	81	109
- Long Severe (26)	13	13
- Long Moderate (39)	14	25
- Long Mild (49)	17	32
- Short Severe (24)	12	12
- Short Moderate (24)	14	10
- Short Mild (28)	11	17
Sham (63)	29	34
Dead (75)	38	37
- Long Severe (41)	23	18
- Long Moderate (8)	1	7
- Long Mild (1)	1	0
- Short Severe (14)	6	8
- Short Moderate (9)	5	4
- Short Mild (2)	2	0

Mortality rate

There was no mortality in Sham pups. Hypoglycemic animals however, had a 28.3% (75/265) mortality rate. Overall there was no significant difference in male and female mortality in any categories. Of the total number of 75 dead animals, 38 were female and 37 male.

As seen in Table 8-1 and 8-2, the severe hypoglycemic animals of either long or short duration, had significantly greater mortality than did either the moderate or mild hypoglycemic animals. In general, there was a direct relationship between mortality and the severity of hypoglycemia.

CATEGORY	MILD	MODERATE	SEVERE
SHORT	2 (2.66%)	9 (12%)a	14 (18.66%)#
LONG	1 (1.33%)	8 (10.66%)b	41 (54.66%)*

Table 8-1. Overall mortality rates of hypoglycemic categories (Fisher Exact Test).

* Significantly different from all other categories; Long Moderate and Long Mild ($p<0.0001$), Short Severe ($p=0.025$), Short Moderate and Short Mild ($p=0.0026$).

Significantly different from Long Moderate ($p=0.048$), Long Mild ($p<0.0001$), and Short Mild ($p=0.004$).

a Significantly different from Long Mild ($p=0.0008$) and Short Mild ($p=0.046$).

b Significantly different from Long Mild ($p=0.0137$).

Category	Long Severe	Long Moderate	Long Mild	Short Severe	Short Moderate	Short Mild	Sham
Mortality	**61.2 %***	**17.02 %#**	**2 %φ**	**36.8 %¶**	**27.3 %Ŧ**	**6.6 %**	**0 %**

Table 8-2. The percentage of mortality within sham and hypoglycemic categories (Fisher Exact Test).

*Significantly different from all other groups ($p<0.0001$).

Significantly different from Long Mild, Short Severe and Sham ($p<0.05$).

φ Significantly different from Short Severe, Short Moderate ($p<0.0001$).

¶ Significantly different from Short Mild and Sham ($p<0.0001$).

Ŧ Significantly different from Short Mild and Sham ($p<0.0001$).

Weight

All animals were weighed at the beginning of the experiments at PD7 and then after the induction of hypoglycemia until PD21. All of the hypoglycemic pups lost weight during the course of hypoglycemia while being away from the dam. They regained their weight after termination of hypoglycemia and returning to their dam. No significant difference was found between the different categories at 7, 15 and 21 postnatal days (Table 9).

	Sham	Long Mild	Long Moderate	Long Severe	Short Mild	Short Moderate	Short Severe
PD7	14.62 ± 0.26	14.82 ± 0.34	14.15 ± 0.28	15.22 ± 0.25	14.87 ± 0.29	14.34 ± 0.42	14.10 ± 0.36
PD15	28.05 ± 0.48	26.73 ± 0.65	25.93 ± 0.60	28.91 ± 0.92	27.13 ± 0.73	26.58 ± 0.59	25.82 ± 0.70
PD21	44.28 ± 0.80	43.62 ± 1.04	42.35 ± 1.35	44.68 ± 1.57	41.30 ± 1.38	42.64 ± 1.30	41.91 ± 1.43

Table 9. Weight before the induction of hypoglycemia (PD7) and during follow up (PD15 & 21)

Behavioral Tests

A minimum number of 24, 18, 14, 11, 12, 12, and 11 rat pups in the sham, long-mild, long-moderate, long-severe, short-mild, short-moderate, and short-severe categories, respectively, were used for behavioral and maturation assessments starting at PD9 - PD14, depending on the test. For open field test at PD21, these numbers were 36, 21, 15, 11, 18, 15, and 11, respectively.

In those pups who survived, early behavioral reflexes as well as open-field testing did not reveal any significant differences ($P < 0.05$) between controls and hypoglycemic animals, regardless of severity or duration of hypoglycemia (Table 10-13).

BEHAVIORAL TEST	Sham	Long Mild	Long Moderate	Long Severe	Short Mild	Short Moderate	Short Severe
Acc. Righting	17.0 ± 0.22	17.0 ± 0.41	16.6 ± 0.46	17.0 ± 0.25	16.4 ± 0.38	17.2 ± 0.35	16.8 ± 0.37
Auditory Startle	13.3 ± 0.13	13.5 ± 0.19	13.4 ± 0.20	13.2 ± 0.30	13.3 ± 0.21	13.4 ± 0.21	13.6 ± 0.28
Eye Opening	16.0 ± 0.12	15.5 ± 0.15	15.9 ± 0.11	15.4 ± 0.31	15.6 ± 0.18	15.8 ± 0.17	15.7 ± 0.14
Forelimb Grasp	9.3 ± 0.11	9.1 ± 0.10	9.1 ± 0.08	9.0 ± 0.00	9.3 ± 0.13	9.2 ± 0.14	9.0 ± 0.00
Hindlimb Grasp	9.2 ± 0.08	9.3 ± 0.16	9.0 ± 0.06	9.1 ± 0.09	9.3 ± 0.14	9.2 ± 0.11	9.0 ± 0.00
Hindlimb Placing	9.7 ± 0.19	9.5 ± 0.16	9.2 ± 0.15	9.4 ± 0.15	9.6 ± 0.30	9.5 ± 0.25	9.2 ± 0.18
Posture	14.6 ± 0.13	16.7 ± 0.22	14.4 ± 0.14	14.4 ± 0.15	14.3 ± 0.19	14.7 ± 0.26	14.6 ± 0.20

Table 10. This table indicates the early behavioral reflexes in each of the hypoglycemic categories by 'Day of Appearance'.

	Sham	Long Mild	Long Moderate	Long Severe	Short Mild	Short Moderate	Short Severe
BAR HOLDING (P16)	28.4 ± 2.55	27.9 ± 3.62	31.6 ± 4.51	26.9 ± 6.23	32.5 ± 5.05	31.3 ± 7.33	28.5 ± 7.62

Table 11. This table indicates bar holding as a test of strength. There was no difference in the duration (seconds) that each of the categories of pups could perform the task.

	Sham	Long Mild	Long Moderate	Long Severe	Short Mild	Short Moderate	Short Severe
RIGHTING (P9)	0.4 ± 0.11	0.09 ± 0.06	0.4 ± 0.16	0.4 ± 0.20	0.1 ± 0.07	0.5 ± 0.17	0.3 ± 0.14

Table 12. This table depicts the ability of rat pups to 'right' themselves. Scores were 0 – less than 2 seconds; 1 – between 2-5 seconds; 2 – greater than 5 seconds.

OPEN FIELD	Sham	Long Mild	Long Moderate	Long Severe	Short Mild	Short Moderate	Short Severe
Grooming	1.1 ± 0.16	1.2 ± 0.20	1.0 ± 0.25	1.2 ± 0.18	0.9 ± 0.19	1.0 ± 0.20	0.8 ± 0.26
Head-lifting	42.4 ± 2.47	41.5 ± 2.72	40.0 ± 3.83	42.6 ± 5.40	42.5 ± 3.70	46.9 ± 2.87	40.8 ± 3.93
Rearing	35.0 ± 2.29	39.5 ± 2.61	39.1 ± 2.60	40.4 ± 4.70	31.4 ± 3.13	33.0 ± 3.98	33.9 ± 3.97
Squares	72.7 ± 4.88	72.5 ± 5.77	68.3 ± 7.15	79.0 ± 8.60	65.7 ± 6.28	64.7 ± 7.64	64.6 ± 7.31

Table 13. Open-field testing on hypoglycemic and sham rat pups at PD21. The findings are depicting as the number of performed behaviors (Mean ± SEM) within a 5 minute time frame.

Neuropathologic And Immunohistochemical Evaluation

FJB at PD9

The areas of brain which were assessed by using FJB staining were: cortex (anterior, posterior and piriform), CA1 (medial and middle sectors), caudate, thalamus, and hypothalamus. Seven long-severe hypoglycemic brains as well as 6 brains in every long-moderate, short-severe, short-moderate, and sham categories were evaluated and compared in the above regions.

Interestingly, the only significant difference between hypoglycemic and sham groups was in a select region of the thalamus, identified as the thalamic reticular nucleus. In this region, there was a significantly higher level of cell degeneration, as depicted by FJB, in the long-severe group compared to sham animals (Figure 10). In order to verify the TRN damage seen in FJB staining, we also stained some of the injured brains with H&E at PD9. As seen in figure 11, cell shrinkage with compacted/condensed nuclei (pyknotic cells) and nuclear fragmentation verified TRN damage of long-severe hypoglycemic brain. The short-severe categories of rat pups also showed an increase in FJB staining, but did not reach statistical significance (Table 14).

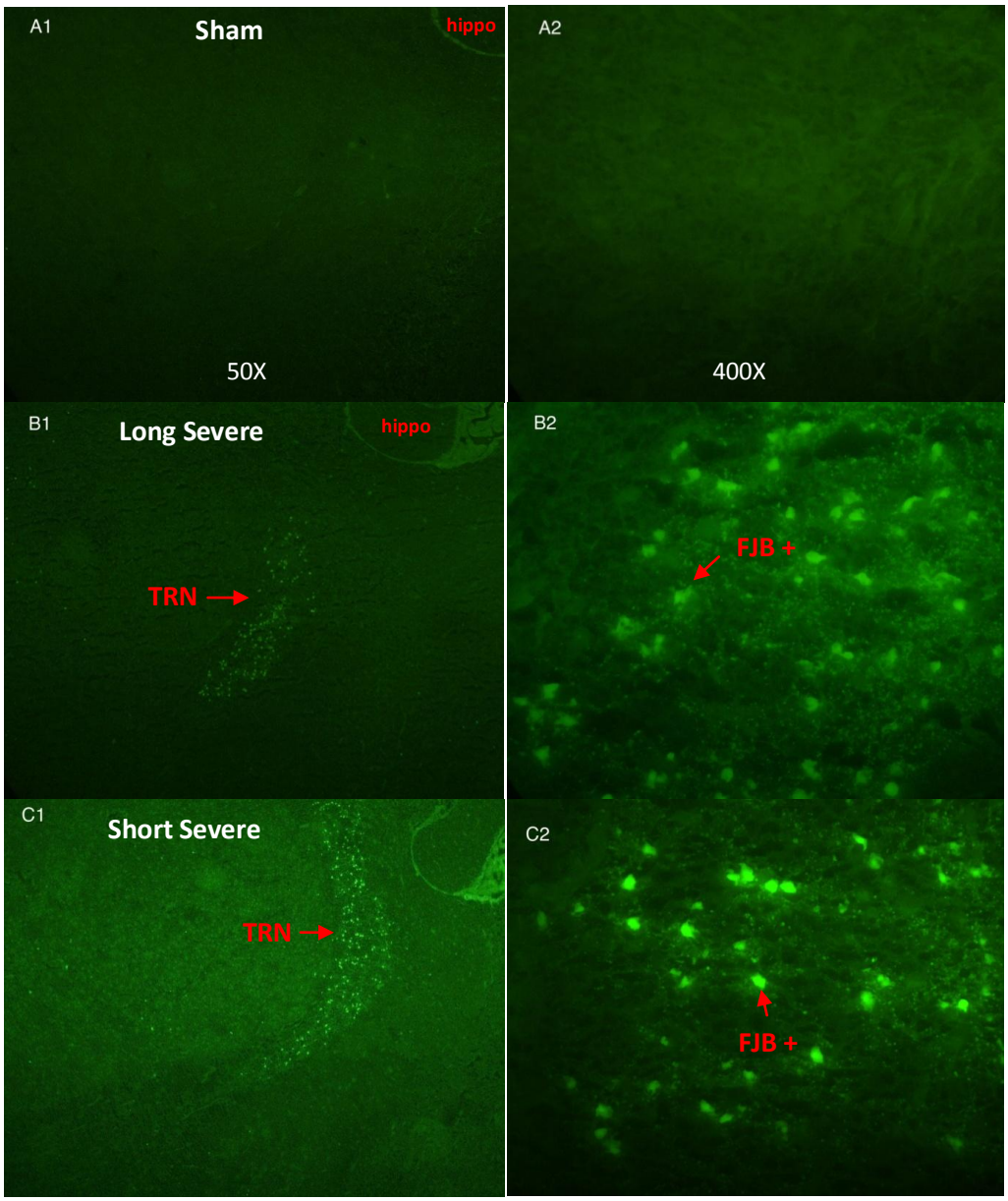


Figure 10. FJB-stained sections illustrating TRN in A (sham), B (long-severe) and C (short-severe) brains at 50X (1) and 400X (2) magnification, respectively.

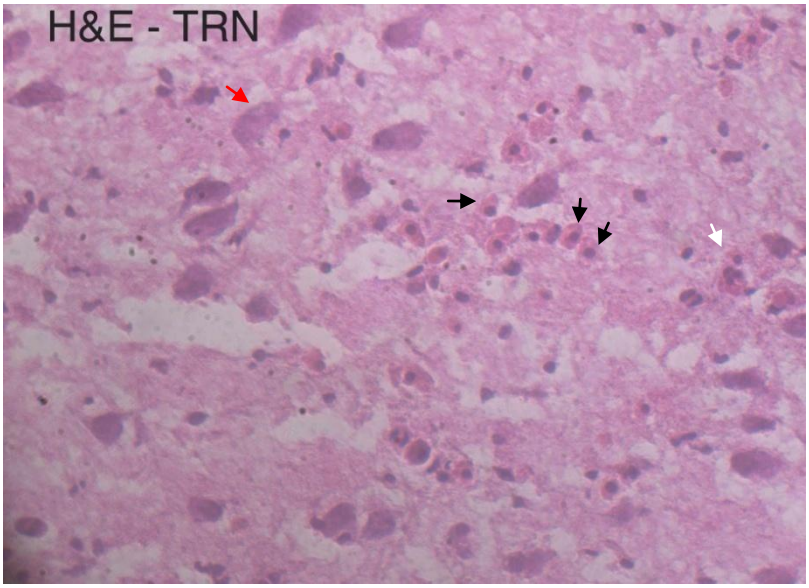


Figure 11. H&E staining showing abnormal cells (pyknotic: black arrows, nucleus fragmentation: white arrow) in TRN of the same long-severe hypoglycemic brain (figure 10 – B2) at 400X magnification. This is consistent with cell death in TRN area detected by FJB. Red arrow shows a normal cell.

FJB	*Long Severe*	*Short Severe*	*Long Moderate*	*Short Moderate*	*Sham*
Cortex-ant	**2.1 ± 0.96**	**1.0 ± 1.00**	**1.3 ± 0.55**	**1.0 ± 0.63**	**1.0 ± 0.51**
Cortex-post	**1.3 ± 0.52**	**0.5 ± 0.50**	**1.5 ± 0.71**	**2.3 ± 0.56**	**6.3 ± 4.62**
Cortex-piriform	**0.8 ± 0.26**	**3.4 ± 1.07**	**1.0 ± 0.51**	**1.5 ± 0.56**	**2.2 ± 0.94**
CA1	**1.3 ± 0.42**	**1.5 ± 0.80**	**2.7 ± 1.60**	**1.2 ± 0.40**	**0.5 ± 0.34**
Caudate	**10.4 ± 4.71**	**12.8 ± 8.96**	**7.0 ± 2.88**	**2.8 ± 1.01**	**8.2 ± 3.34**
Thalamus	**10.0 ± 0.75**	**5.8 ± 1.42**	**4.7 ± 1.38**	**2.2 ± 0.70 ***	**6.7 ± 0.80**
TRN	**48.1 ± 16.03 ***	**32.8 ± 14.60**	**5.5 ± 3.45**	**4.3 ± 1.96**	**1.8 ± 0.87**
Hypothalamus	**4.7 ± 1.56**	**5.3 ± 1.76**	**6.8 ± 1.74**	**1.8 ± 0.48**	**5.3 ± 1.49**

Table 14. The number of degenerating neurons in different regions of the brain (the numbers in CA1 of hippocampus represent the total number of degenerating cells in medial and middle sectors of CA1). (numbers represent Mean ± SEM)

*significantly different from Sham ($p<0.05$).

Immunohistochemistry

GFAP at PD21

Six PD21 long-severe hypoglycemic brains were stained and assessed with GFAP and compared with 6 brains in sham group. CA1 of hippocampus and cortex (anterior and posterior) were evaluated in GFAP-stained brains. Figure 12 shows that GFAP+ cells displayed activated astrocyte morphology, with short processes and swollen cell bodies at PD21.

As indicated in Table 15, there was no significant increase in GFAP immunopositive reactive astrocytes in the hypoglycemic brains and the number of astrocytes did not increase in the CA1 and cortex compared to sham brains.

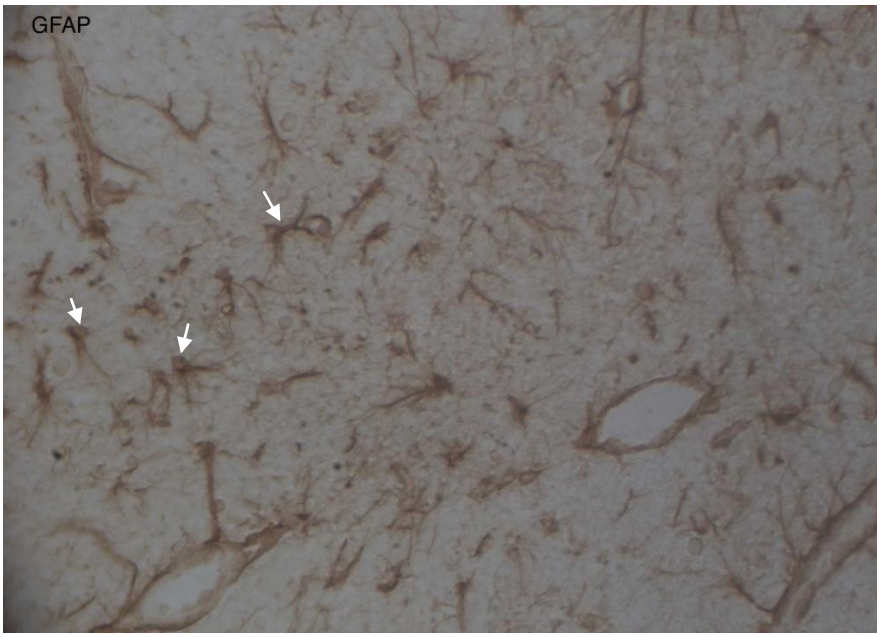


Figure 12. GFAP staining from CA1 of hippocampus of a PD21 hypoglycemic rat brain showing astrocytic infiltration. The arrows indicate reactive astrocytes with swollen cell bodies and short, thick processes.

GFAP	cortex (anterior)	cortex (posterior)	CA1
Long Severe (n = 6)	10.75 ± 1.10	12.50 ± 1.42	17.33 ± 2.62
Sham (n = 6)	10.83 ± 1.15	10.08 ± 1.05	19.17 ± 1.24

Table 15. The average number of astrocytes in medial and middle sectors of CA1, and two spots of cortex in both anterior and posterior sections. (numbers represent Mean ± SEM)

MBP at PD21

Six rat pup brains in every long-severe and sham categories were MBP stained and evaluated by using densitometric analysis in corpus callosum (anterior and posterior) and reticular nucleus of thalamus (TRN) (Figure 13).

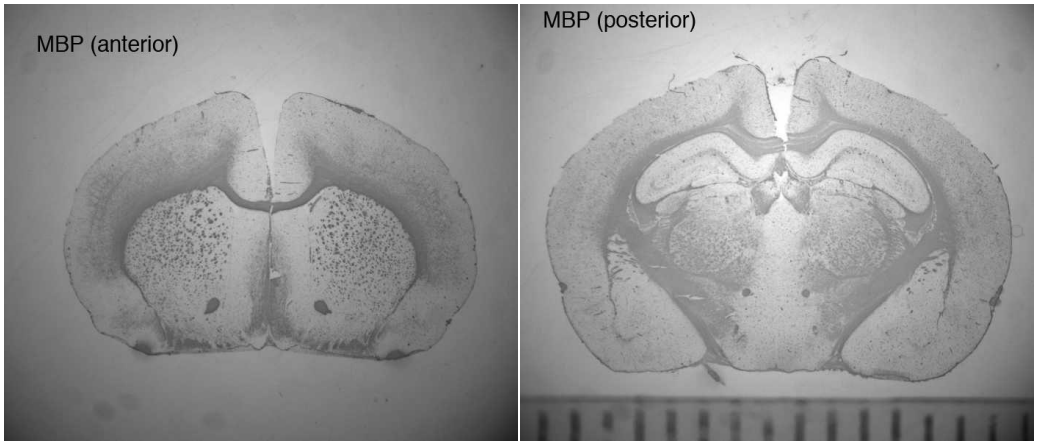


Figure 13. MBP staining of anterior (left) and posterior (right) sections showing white matter tracts.

No marked reductions in MBP densitometry was elicited in the assessed regions of hypoglycemic brains compared to sham brains (Table 16).

MBP Densitometry	CC (anterior)	CC (posterior)	TRN
Long Severe (n = 6)	0.25 ± 0.03	0.20 ± 0.02	0.27 ± 0.01
Sham (n = 6)	0.25 ± 0.01	0.18 ± 0.01	0.25 ± 0.01

Table 16. MBP densitometric values in corpus callosum and TRN. Numbers represent Mean ± SEM.

Neu-N at PD21

Six long-severe hypoglycemic PD21 rat brains were stained with Neu-N and compared with 6 sham brains in both CA1 and TRN regions of the brain. The nuclei were counted in 3 regions of CA1 as well as TRN (Figure 14).

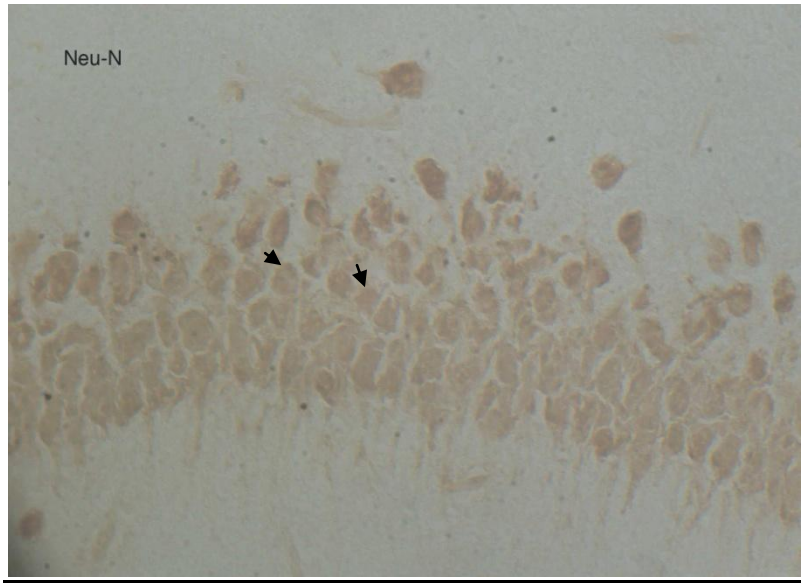


Figure 14. Neu-N staining from middle sector of CA1 of hippocampus in one long-severe hypoglycemic PD21 rat pup at 400X magnification indicating nuclei (arrows).

As Table 17 shows, no significant difference was found in Neu-N counts at PD21 in either regions between two categories.

Neu-N	CA1	TRN
Long Severe (n = 6)	87.61 ± 1.28	54.50 ± 4.75
Sham (n = 6)	89.61 ± 1.15	51.17 ± 3.47

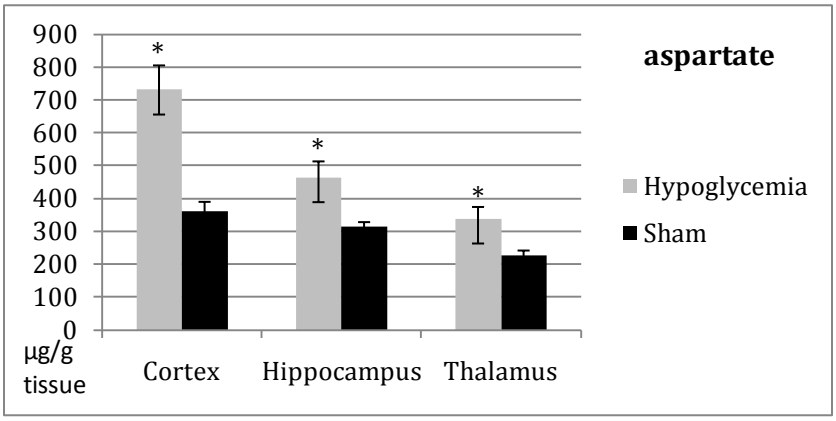
Table 17. The average number of nuclei in medial, middle and lateral sectors of CA1 of hippocampus and TRN. Numbers represent mean ± SEM.

Neurochemical Assessment

A total of twelve PD8 brains (6 Long-Severe hypoglycemic and 6 Sham) were measured for the level of excitatory amino acids (EAA) as well as matrix metalloproteinases (MMPs). Rat pups were prepared and treated as described above. Following ≥12 hours of hypoglycemia, rat pups were immediately sacrificed by decapitation, and their brains dissected fresh, identifying 3 major regions. The dorsolateral cortex, hippocampus and thalamus. These regions were then rapidly frozen in dry ice and stored at -70 °C for biochemical determination.

Excitatory Amino Acids

Glutamate, GABA, Glutamine, Aspartate, L-Serine, D-Serine, Glycine, Arginine, Taurine, Alanine were measured by using HPLC. Hypoglycemic pups had significantly lower concentrations of alanine, and glutamine, and higher levels of aspartate in all three regions of their brain. Arginine concentration was remarkably higher in hippocampus and thalamus only. Other amino acids did not show any significant differences between hypoglycemic and sham animals (Figure 15 and Table 18).


aspartate
900
800
700
600
500
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300
200
100
0
µg/g tissue
Cortex
Hippocampus
Thalamus
Hypoglycemia
Sham
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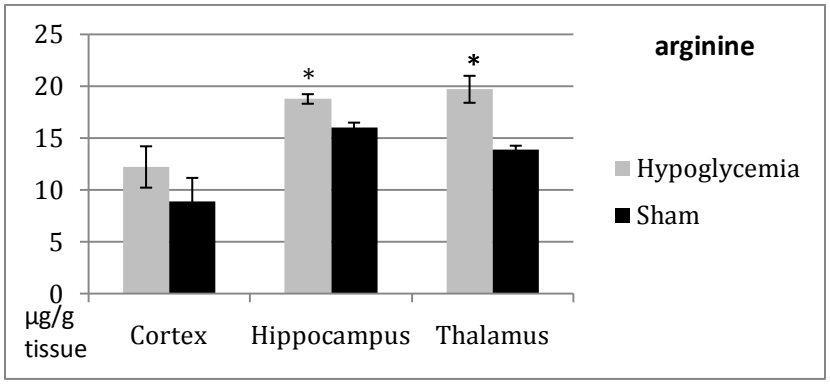
arginine
25
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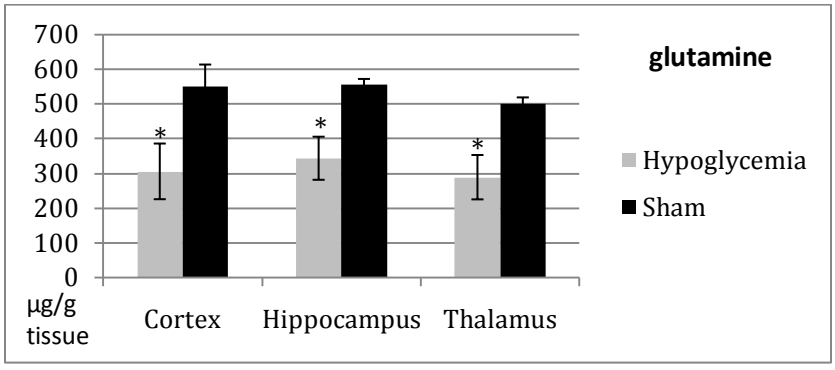
glutamine
700
600
500
400
300
200
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0
µg/g tissue
Cortex
Hippocampus
Thalamus
Hypoglycemia
Sham
*
*
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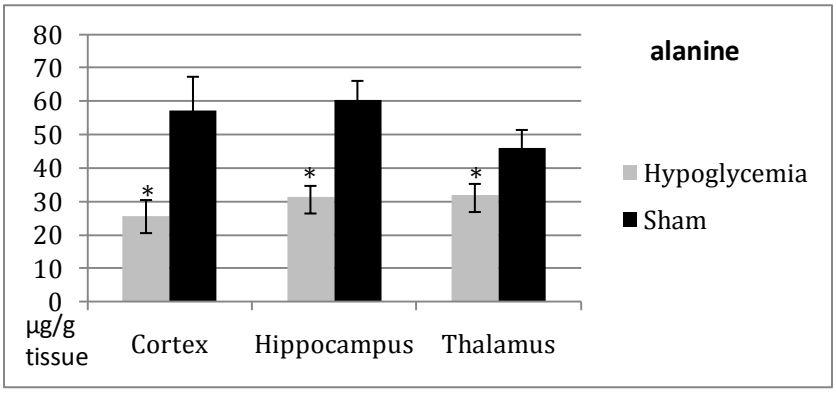
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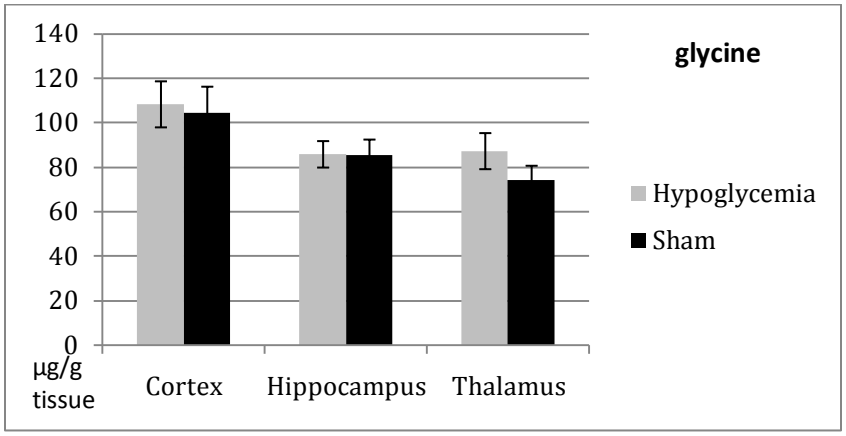
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µg/g tissue
Cortex
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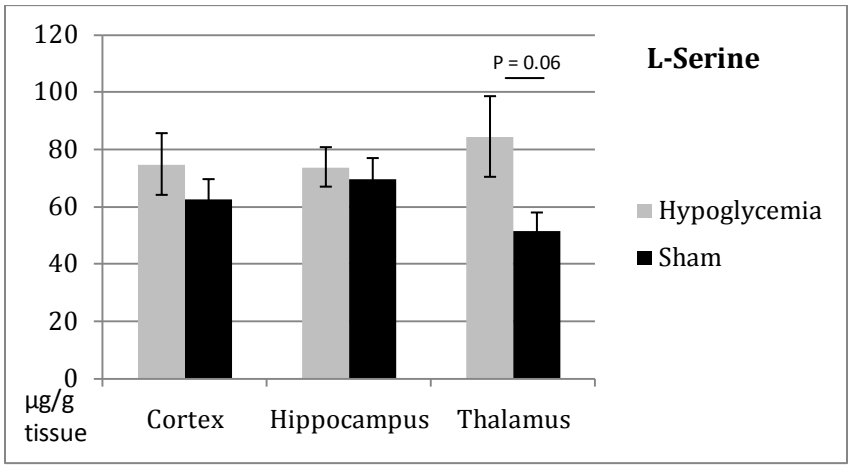
Hypoglycemia
Sham


L-Serine
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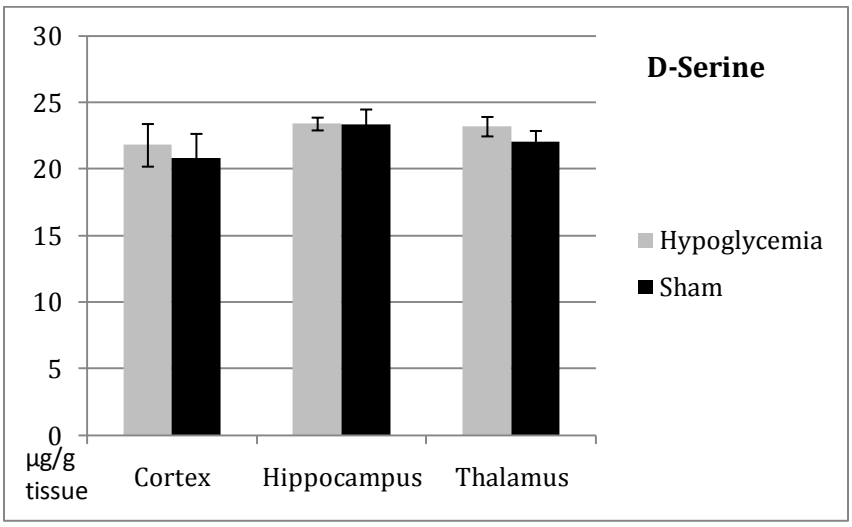
µg/g tissue
Cortex
Hippocampus
Thalamus
Hypoglycemia
Sham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20
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10
5
0
µg/g tissue
Cortex
Hippocampus
Thalamus
Hypoglycemia
Sham

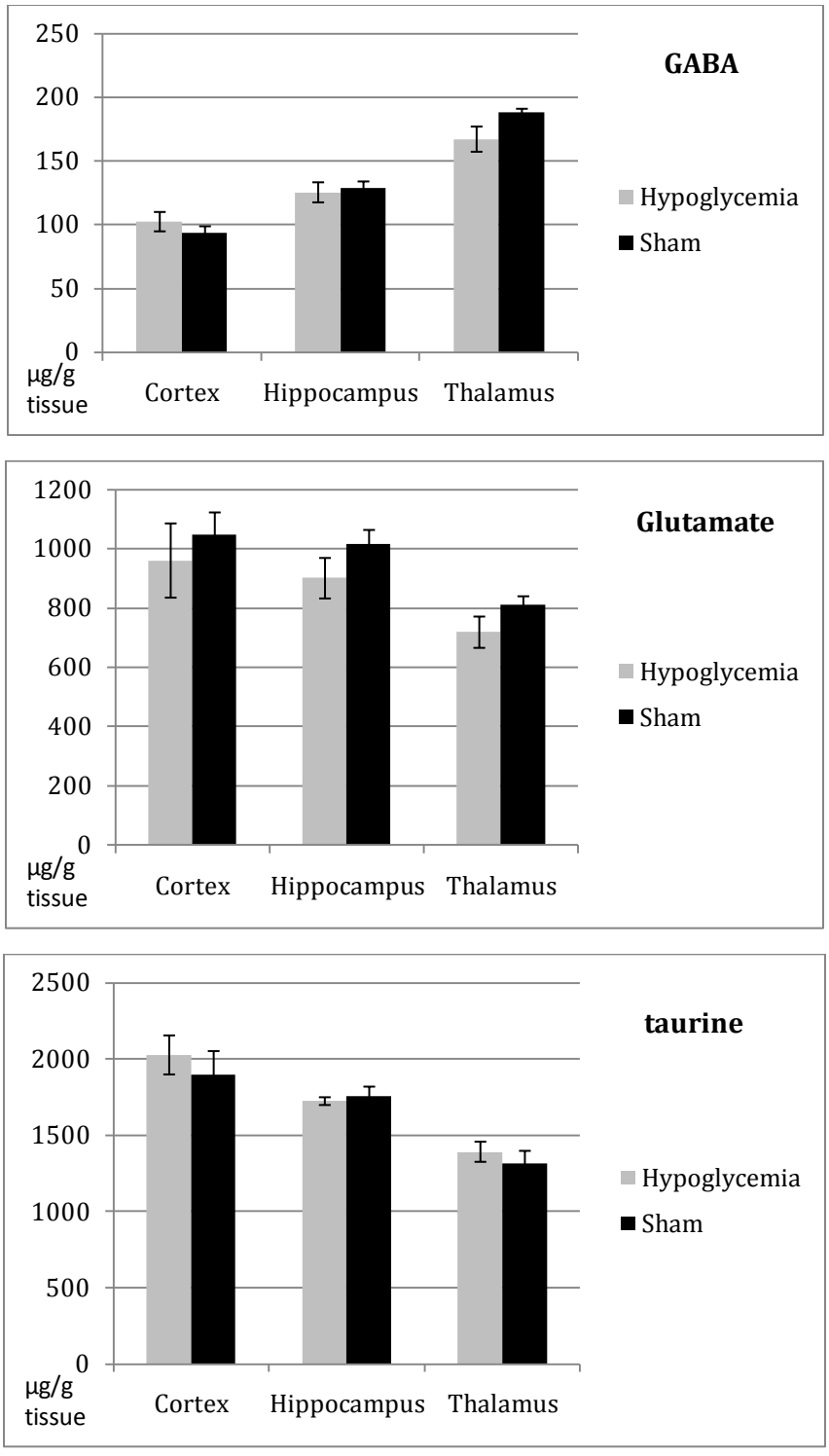

Figure 15. Amino acid concentrations in cortex, hippocampus and thalamus of sham versus long-severe hypoglycemic PD8-9 rat pups. (*Significantly different from sham)

	Cortex		Hippocampus		Thalamus	
	Hypoglycemia	Sham	Hypoglycemia	Sham	Hypoglycemia	Sham
Alanine	25.6 ± 4.9 *	57.1± 10.3	31.5 ± 3.3 *	60.3 ± 5.8	31.9 ± 3.5 *	46.1 ± 5.3
Aspartate	731.4 ± 74.8 *	362.2 ± 28.6	464.6 ± 49.4 *	313.5 ± 15.6	338.8 ± 36.5 *	226.7 ± 15.9
Glutamine	305.1 ± 79.9 *	550.4 ± 61.6	342.8 ± 61.9 *	554.7 ± 15.9	288.4 ± 63.7 *	499.2 ± 18.4
Arginine	12.2 ± 1.9	8.9 ± 2.3	18.8 ± 0.46 *	15.9 ± 0.5	19.7 ± 1.3 *	13.9 ± 0.4
Glycine	108.3 ± 10.3	104.4 ± 11.8	85.8 ± 5.9	85.3 ± 7.1	87.3 ± 8.1	74.4 ± 6.3
L-Serine	74.8 ± 10.8	62.5 ± 7.02	73.7 ± 6.9	69.5 ± 7.3	84.4 ± 14.08	51.5 ± 6.3
D-Serine	21.8 ± 1.6	20.8 ± 1.8	23.4 ± 0.5	23.3 ± 1.1	23.2 ± 0.7	22.01 ± 0.9
GABA	102.4 ± 7.6	93.6 ± 5.2	125.4 ± 7.9	128.9 ± 5.03	167.1 ± 9.9	188 ± 2.9
Glutamate	960.6 ± 125.1	1046 ± 76.8	901 ± 68.3	1017 ± 46.7	718.9 ± 52.8	811.1 ± 28.9
Taurine	2026 ± 128	1899 ± 152.2	1723 ± 25.5	1755 ± 63.1	1391 ± 66.1	1315 ± 81.9

Table 18. Amino acid concentrations (µg/g brain tissue) in cortex, hippocampus and thalamus (numbers represent Mean ± SEM) (*significantly different from sham)

Matrix Metalloproteinases and Redox Ratio

In the brain tissue, MMP-2 was detected as the gelatinolytic activity at 72 KDa on zymography. However, MMP-9, the gelatinolytic activity at 92 KDa, was below detectable limit in most tissue samples. Hypoglycemic rat pups had significantly higher activity of MMP-2 in cortex, hippocampus, and thalamus compared to shams (Figure 16-A). The redox ratio did not show remarkable difference between hypoglycemic and sham animals in different regions of the brain (figure 16-B), though there were significant differences when whole brain areas were compared.

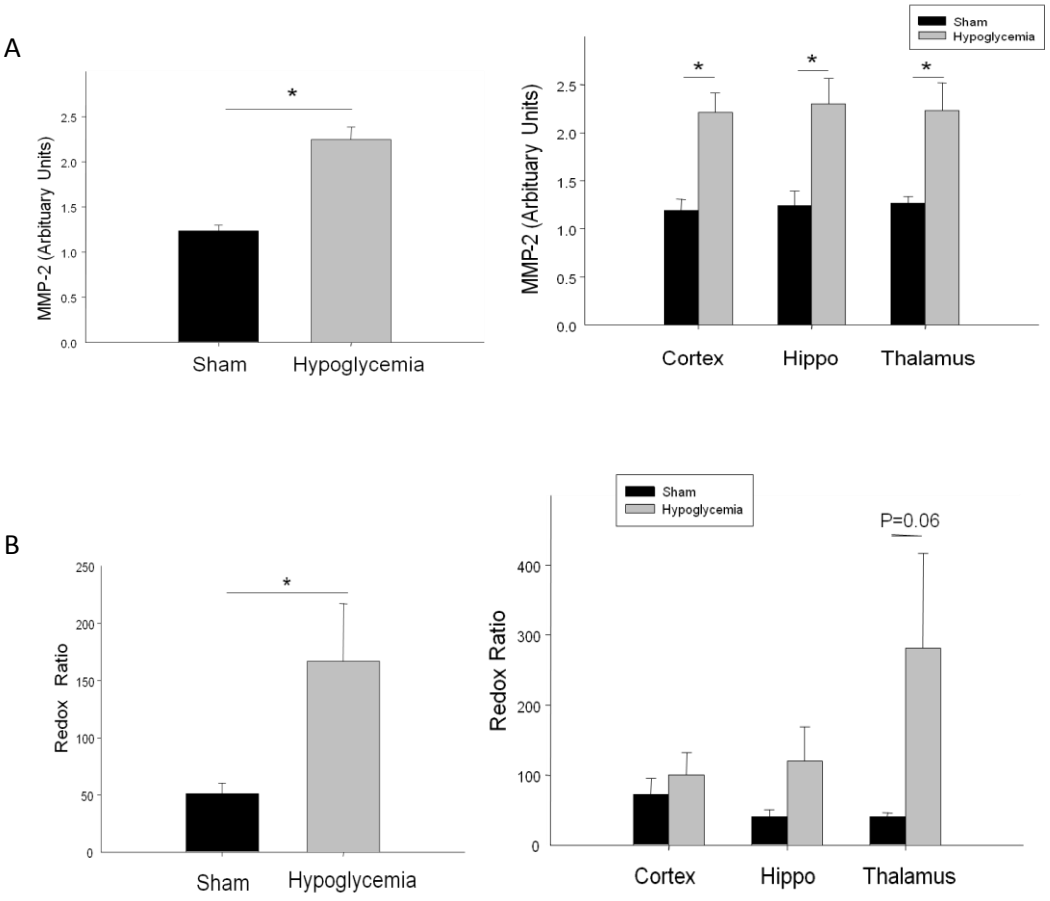

Figure 16. Activity of MMP-2 (A) and Redox Ratio (B) in cortex, hippocampus and thalamus of sham versus long-severe hypoglycemic PD8-9 rats. (* significantly different from sham)

Discussion

Despite all studies regarding the effects of hypoglycemia on functional and pathological outcome of the newborn, the critical question still remains as to whether pure hypoglycemia contributes to damage in the immature brain. The purpose of this study was to determine the effects of isolated hypoglycemia by assessing neuropathologic, neurodevelopmental, and neurochemical outcomes. Our findings indicate that severe hypoglycemia, defined as being less than 1.1 mmol/L and with duration of greater than 12 hours, in the newborn rat pup, a) Caused biochemical alterations diffusely throughout the cortical regions of the brain, b) Caused cell death in a specific region of the thalamus, known as the thalamic reticular nucleus, however c) these alterations did not result in early behavioral abnormalities, and d) did not appear to cause permanent brain damage in the newborn rat pup. Whether long term neurodevelopmental or neuropathologic alterations would result is the subject of ongoing investigations.

Mortality, Gender, and Weight

Weight was not involved in hypoglycemia as there was no significant weight difference between the shams and different categories of hypoglycemic rats at different ages. Gender did not appear to be significant in our study, neither on the severity of hypoglycemia nor on the death rate. The similar mortality in both genders indicates the same tolerance of male and female pups to hypoglycemia. Behavioral and

neuropathologic evaluations did not show any significant difference between genders.

There was no death in the sham animals. For the hypoglycemic groups there was a direct relationship between mortality and the severity of hypoglycemia since highest mortality was observed for the severe hypoglycemic group for both long (54.7%) and short (18.7%) duration. Moderate and mild hypoglycemic groups had lower mortality, with only 4% in the mild category. The exact cause of death is not known but we observed that all dead animals were pale at time of death, suggesting that death may have been due to cardiac compromise, as opposed to brain death. This could be determined by looking at blood pressure or heart rate in animals during the course of hypoglycemia as indicators of blood flow. We did not assess these vital signs because of limitations and technical difficulties in such small pups such as inability to restrain the rat pups and difficulties with fixing the wrapped sphygmomanometer cuff around their tiny tails during the induction of hypoglycemia. Therefore, the potential occurrence of hypotension during severe hypoglycemia cannot be ruled out. However, whereas hypotension as a complication of severe hypoglycemia leads to enhanced energy failure, it does not exaggerate neuronal injury (Auer, Hall et al. 1986). The higher mortality in our hypoglycemic animals also raises the question as to whether a larger percentage of this group would have shown pathologic or biochemical abnormalities, had they survived. Indeed, the underlying question of our

research was to determine whether hypoglycemia, in the absence of cardiac or other compromise might cause brain injury. Given the pallor appearance of the animals at the time of death, one might presume that blood pressure was compromised, and our assessment of isolated hypoglycemia on brain injury remains correct.

Neuropathology

Pathologic pattern of brain injury due to isolated hypoglycemia has often been examined in adults (Auer 1986) but studies in neonates are limited. Acute hypoglycemia is associated with neuronal injury in adult human and rodent brains. The cerebral cortex, hippocampus (particularly CA1 and dentate gyrus), and caudate have been shown to be the most vulnerable areas of the brain to hypoglycemic insult in adult rats (Sandberg, Butcher et al. 1986; Ennis, Tran et al. 2008). Only a few studies have looked at the effects of acute hypoglycemia in developing brain. Yamada et al. showed brain damage in the cerebral cortex of PD25 hypoglycemic developing rats (Yamada, Rensing et al. 2005). Apoptotic cell injury has been demonstrated in the striatum and hippocampus of developing mice brain subjected to 4 hours of acute hypoglycemia (Kim, Yu et al. 2005). Ennis et al. studied the effects of moderate to severe hypoglycemia on PD7 rat brain by using FJB and did not find any association between hypoglycemia and brain cell injury at this age (Ennis, Tran et al. 2008). In a study by Anderson et al. autopsy specimens of 6 hypoglycemic human infants were examined. Three infants had died with

untreated hypoglycemia while the others had successfully been treated for their hypoglycemia but died of other causes. In those that died with untreated hypoglycemia, the most severely affected area was the occipital cortex (Anderson, Milner et al. 1967). MRI studies have supported this pathologic finding, showing a diffuse cortical and subcortical pattern of injury within the parieto-occipital lobes as the most severely affected region of hypoglycemic infants (Barkovich, Ali et al. 1998; Traill, Squier et al. 1998; Murakami, Yamashita et al. 1999; Caraballo, Sakr et al. 2004).

Brain injury resulting from hypoglycemia is directly dependent on the availability of metabolites such as glucose and lactate, required for energy production. Regions of the immature brain that have higher metabolic demands due to rapid development would be therefore more vulnerable to damage. In human newborns, damage is observed in the parieto-occipital white matter region perhaps because of the rapid development of vision in the human newborn. In rat pups, however, other regions of the brain may be more vulnerable. Vision in newborn rat pups does not appear to develop until much later as they do not even open their eyes until PD15-16. The sense of smell or touch has been shown to be rapidly developing in these animals at PD7, therefore, regions of the brain responsible for these senses would be more susceptible to hypoglycemic brain injury, due to their high metabolic demands.

In our study, in addition to the evaluation of the susceptible regions of adult and developing rodent brain to hypoglycemic insult found in other

studies (hippocampus, striatum, and cerebral cortex) (Auer, Olsson et al. 1984; Kim, Yu et al. 2005; Yamada, Rensing et al. 2005; Suh, Hamby et al. 2007; Ennis, Tran et al. 2008), we also looked at the lateral orbital area which is a part of piriform cortex and represents olfactory area of the rat brain (Kolb and Tees 1990). Based on the previous studies performed in our lab regarding the number of animals required for this type of study, six or seven rat pups from each category were used for our histological assessments.

In pathologic evaluations for PD9 animals, we found transient brain injury in TRN of the severely hypoglycemic animals; however, other regions of the brain appeared to be spared. Comparable numbers of FJB+ cells were found in different regions of sham and hypoglycemic brains suggesting that acute hypoglycemia was not associated with neuronal injury at this age, except in the reticular nucleus of thalamus (TRN). The FJB+ cells at this age likely reflect the programmed cell death that is active until PD10 (Rice and Barone 2000; White and Barone 2001; Ennis, Tran et al. 2008). The tolerance of different regions of the immature rat brain to hypoglycemia in our study may be interpreted by preservation of high-energy phosphate reserves during hypoglycemia via utilizing alternate substrate, lactate, by immature brain as described in other studies (Hernandez, Vannucci et al. 1980; Vannucci, Nardis et al. 1981; Mujsce, Christensen et al. 1989; Yager 2002).

In the TRN, however, the increased number of FJB+ degenerating cells reflects hypoglycemia-induced brain injury in a region specific manner. The TRN is part of the ventral thalamus and is separated from the thalamus by the external medullary lamina (Figure 6-b – rectangle). As projections to the cortex from the thalamus must pass through the TRN, it is believed that this complex acts as a "gateway" (Figure 17). In the rat, It has been suggested that the internal attentional searchlight (selective attention) - the ability to focus and concentrate on a given object, stimulus, event, thought, or activity while excluding competing stimuli - is controlled by GABAergic cells of the TRN. In other words, the reticular complex limits the number of subjects the individual can pay attention to at any one time and controls which sensory inputs are the subject of attention of the cerebral cortex. The expression of the searchlight is the production of rapid bursts of firing in a subset of thalamic neurons (Crick 1984).

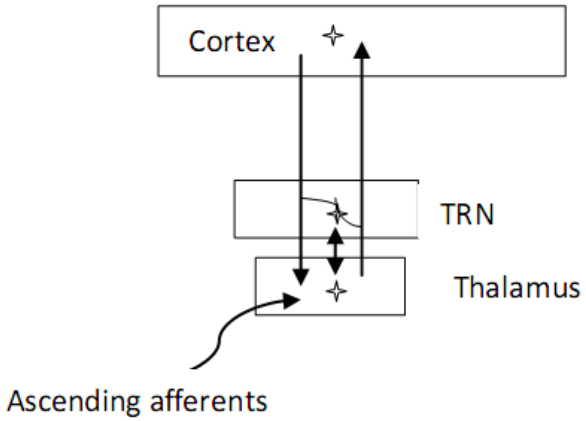

Figure 17. The thalamic reticular nucleus, that is believed to function to "gate" signals to the cerebrum from the thalamus, receives input from the ipsilateral cerebral cortex and dorsal thalamic nuclei. In rat, primary thalamic reticular nucleus efferent fibers project to the nuclei of the dorsal and anterior thalamus, but never to the cerebral cortex (Gonzalo-Ruiz and Lieberman 1995).

The vulnerability of TRN to damage in hypoglycemic conditions may be due to higher metabolic demands of this nucleus of the thalamus in the developing rat brain. This means that when TRN energy demand outstrips supply in hypoglycemic immature brain, the TRN cells are degenerated. Our *severe* hypoglycemic animals, who showed greater numbers of FJB+ cells, may reflect the higher energy demands of TRN in rat pups. Increased rCBF in different areas of hypoglycemic newborn dog brain particularly in thalamus in Mujsce et al. study may also reflect higher energy demands of this nucleus of brain (Mujsce, Christensen et al. 1989). Measuring CMRglucose in TRN and other regions of brain might also help to determine the differences in metabolic demands of different regions of immature brain and the mechanism of damage in this specific nucleus of

thalamus. Considering the function of TRN (selective attention), high activity of this nucleus of the thalamus may be explained by the need of the developing rat pups to exclude excess sensory stimuli to efficiently track their mothers for feeding or maintaining temperature by using their somatosensory system which is rapidly developing at this age. In other words, TRN may act as a sensory filter and spotlight area allowing only behaviorally relevant information to capture limited attentional resources to enable the rat pup to attend selectively to stimuli that signal danger or opportunity.

One might question the inconsistency between the lack of brain injury in PD7 rats in Ennis study (Ennis, Tran et al. 2008) and TRN damage found in our PD9 hypoglycemic brains. A possible explanation might be due to the increased severity and duration of hypoglycemia induced in the current study. In this regard, hypoglycemia was only induced for 3.5 hours and to a level of 1.6 mmol/L, significantly less severe than in our study. Had we taken the same approach, it is likely we would also not have found any damage, given that no damage was seen in our moderate or mild groups of hypoglycemic animals.

It is important to mention that not all the hypoglycemic pups in the long-severe category had TRN brain damage. Of the 7 long-severe hypoglycemic rats, only 4 of them showed TRN brain injury while the others did not. In the short-severe category, also, TRN damage was not detected in all animals. We speculate that this may have been due to

several possible factors. Since our glucometer did not measure blood sugar concentrations below 1.1 mmol/L, it is possible that there was variability in glucose concentrations, even in our severe group, and that only the lowest showed FJ-B positivity. Alternatively, because this is a 'metabolic' injury, there may be differences between animals as to their 'supply and demand' requirements for glucose, those with higher demands having a greater susceptibility to damage. Unfortunately, we were not able to detect the blood glucose concentrations of <1.1 mmol/l in our experiments since we had to sacrifice the pups to obtain more amount of blood required for this purpose. It should be emphasized however, that none of those animals with concentrations of blood glucose above 1.1 mmol/L showed an increase in FJ-B positive cells above control.

In order to determine whether the brain damage found in long-severe hypoglycemic brains at PD9 was persistent or transient in nature, brains at PD21 were also stained with Neu-N (neuronal), GFAP (astrocytic) and MBP (myelin). The number of Neu-N positive cells revealed no significant neuronal loss in the brains of the hypoglycemic animals. Moreover, activated astrocytes, which are expected to infiltrate the injured region of the brain, were not increased in hypoglycemic animals. Densitometric analysis of MBP-stained brain sections did not show differences between long-severe hypoglycemic and sham rats. This indicates there was no white matter injury or oligodendroglial dysfunction as a result of hypoglycemia. Overall, the lack of brain injury in late pathological

assessments (PD21), even in long-severe hypoglycemic animals, may suggest that despite early pathologic alterations and TRN brain damage at PD9, hypoglycemic insult caused no persistent or overt permanent injury.

The absence of permanent injury may rule out the process of necrosis in which the damage is permanent and unrecoverable and be explained by either apoptosis or neurogenesis. We hypothesized that the degenerating cells seen in TRN at PD9 may be those that are undergoing delayed programmed cell death during the rapid developmental stage of the immature rat brain. This means that the dead cells found in TRN of the hypoglycemic rat brains are those that would have died anyway due to the normal process of apoptosis and hypoglycemia just expedited this process. An alternate hypothesis may be that because the brain is rapidly developing at this stage of animal's life, the degenerated cells start to be regenerated after hypoglycemic insult and this is why the TRN damage is transient and not seen at PD21. Having said these, we do not have any direct evidence of these assumptions at the present time.

Lack of neuronal loss per se in PD21 brains of our study, however, does not necessarily mean that hypoglycemia does not change neuronal structure and/or connectivity. The cells in nervous tissue are densely packed and little information on their structures and interconnections can be obtained if all the cells are stained. Axons and dendrites (filamentary extensions of the neuronal cells) are too transparent and slender to be seen with normal staining methods. Therefore, further studies are

required to assess more detailed structures of the neurons such as golgi's method and synaptophysin. Golgi's technique, which is used for distinguishing morphology at the individual neuron level, stains a limited number of cells at random in their entirety. Dendrites, as well as the cell body are clearly stained and can be followed in their entire length. This allows one to track connections between neurons and to make visible the complex networking structure of many parts of the brain and spinal cord (Nicholls 2001). Synaptophysin (a protein involved in neurotransmitter exocytosis) immunostaining is also another method for looking at more detailed structures of nervous tissue and quantification of synapses. Increased synaptophysin reflects increased number of synapses and therefore might be worthwhile to be performed to evaluate our hypoglycemic rat brain samples (Calhoun, Jucker et al. 1996).

Early Reflexes and Behavioral Tests

Clinical and neurologic outcomes after neonatal hypoglycemia, especially symptomatic and recurrent hypoglycemia, have been studied for many years. Burns et al. studied 35 term infants with early brain MRI scans after symptomatic neonatal hypoglycemia without evidence of hypoxic-ischemic encephalopathy, and then assessed neurodevelopmental outcomes at a minimum of 18 months. They found white matter injury which was not confined to the posterior regions. Hemorrhage abnormalities in basal ganglia and thalamus, middle cerebral artery infarction, and cortical involvement were also seen. These children

had relatively common poor cognition and seizures at 2 years of age. Other abnormalities found in these infants were motor disabilities such as cerebral palsy (CP), speech and language delays, suboptimal head growth, and vision abnormalities (Burns, Rutherford et al. 2008). Yalnizoglu et al. studied long-term prognosis of neonatal hypoglycemia in patients with MRI pattern of damage typical for neonatal hypoglycemia who had prenatal and/or perinatal problems including IUGR, HI, indirect hyperbilirubinemia and sepsis. The neurologic sequelae in these children, even though not necessarily due to isolated hypoglycemia, were epilepsy, developmental delays, learning and behavior difficulties, attention deficit hyperactivity disorder (ADHD), microcephaly, autism and cortical blindness (Yalnizoglu, Haliloglu et al. 2007).

We performed early reflexes and open field tests to assess rat's brain development because as the animal grows, maturational and neurologic alterations in the brain are reflected in the animal's behavior. Of course, it is important to consider that the animal's behavior is also affected by environmental and maternal parameters such as feeding, stress, home-cage or dam separation, temperature, light, and caretaker (Mccall, Lester et al. 1969; Kolb and Gibb 1991; Schallert, Woodlee et al. 2003). Therefore, it is essential to avoid these external variables in order to obtain reliable results.

We were aware that pure reflex tests, which are not dependent on size or weight and are also not confounded by learning or handling, can

provide reliable comparisons between groups. We therefore chose to perform tests such as placing and grasping. These tests were positive from the beginning of testing at PD9 and remained positive in both sham and hypoglycemic animals. As weight played no significant role in our study, behavioral tests requiring agility and physical strength including bar holding, and righting were also valuable for comparison. Pups in all groups were able to right within 2 seconds at PD9 and showed no difference in motor coordination and body strength in bar holding test. They all were able to open both eyes at PD15-16 and react to auditory startle at PD13. They also developed a normal posture, an indicator of maturation, at PD14-15 and performed a positive acceleration righting test (as statokinetic labyrinthine response) at PD16-17.

In open field test, four different behaviors were assessed within a 5 minute time frame; ambulation, rearing, head-lifting and grooming (Lubics, Reglodi et al. 2005; Kiss, Hauser et al. 2007). These behaviors were videotaped and scored by a blinded observer. Motor activity was expressed as a total number of squares crossed (with all four feet on one square) during the whole period of testing. Rearing is one reliable index of activity (Ivinskis 1968). Combined with ambulation it is an index of "nonspecific excitability level" (Walsh and Cummins 1976). Head-lifting is generally taken as an index of exploration as well as a useful co-index for nonspecific excitability. Grooming, although it has a relatively low reliability (Ivinskis 1968), is negatively related to indexes of high-activity

states (Walsh and Cummins 1976) and when the animal is stressed, less grooming is observed.

The evaluation of early reflexes and open field testing in shams and different categories of hypoglycemic animals from PD9 to PD21 showed no neurological deficits in the hypoglycemic animals in the short term. There was no effect of hypoglycemia on the physical maturation, sensory motor skills, muscular strength, motor coordination, locomotor activity, exploration and cognitive function of the rat animal. However, our behavioral evaluations were performed within a limited period of the animal's age; from nine days to three weeks. Therefore, longer term behavioral follow up might help us to determine neurodevelopmental and neurologic deficits that neonatal hypoglycemia may cause.

Presence of TRN damage at PD9 and negative behavioral outcomes do not necessarily contradict each other. As mentioned before, the most regions of rat brain in our study were spared and the subtle transient brain injury in TRN may not be enough to cause behavioral impairments. Also, since the pattern of damage was in a region specific manner and confined to the thalamic reticular nucleus in our study, those behavioral tests assessing the function of this area (selective attention) might help us to find out the possible short or long term neurological outcomes of hypoglycemia on the immature rat brain. As in the development of our protocol, behavioral test were performed first followed by pathology, it was not possible to perform these behaviors. However,

Prepulse Startle Inhibition, Water Maze, Object Recognition, or Radial Arm Maze are the behavioral tests that might help us in this regard.

Neurochemical Alterations

Excitatory Amino Acid (EAA) Release during Hypoglycemia

The concentrations of EAAs in tissue from the cortex, hippocampus, and thalamus were measured. The amino acid concentrations showed alterations in the PD8-9 long-severe hypoglycemic rat brains, however, the only significant alterations were found in the concentration of aspartate, arginine, glutamine, and alanine. There was a significant increase in aspartate and decrease in glutamine, and alanine in all three regions of the hypoglycemic rat brains. Arginine, however, was only increased in the hippocampus and thalamus, and not in the cerebral cortex. Evaluation of more number of animals might show significant increase or decrease of other amino acids in different regions of hypoglycemic rat brains including an increase of arginine in the cerebral cortex as well.

There is very little information about biochemical alterations resulting from hypoglycemia in the immature brain. However, there have been some studies about insulin-induced hypoglycemia on adult animals that might assist us in understanding the perturbations that occur in the immature brain. Studies reveal that in adult animals the mechanism of hypoglycemic neuronal death is similar to that of hypoxia-ischemia.

Hypoglycemia causes neuronal death not merely by starvation but rather through an active mechanism. Severe hypoglycemia (<1 mmol/L) causes an abrupt energy failure. This in turn causes a cascade of events that eventually lead to neuronal cell death (Suh, Hamby et al. 2007). Hypoglycemia induces neuronal depolarization leading to release of excitatory amino acids and zinc. Excessive aspartate and glutamate are released into the extracellular space and activate excitatory amino acid receptors (NMDA). Consequently, calcium and/or zinc influxes take place, ROS are produced from mitochondria or NADPH oxidase, DNA is damaged, and membrane breaks in the cell lead rapidly to neuronal death (Figure 18). Other neurochemical alterations include energy depletion, enzyme activation (phospholipases and proteases), tissue alkalosis, and a tendency for all cellular redox systems to shift towards oxidation (Sandberg, Butcher et al. 1986; Butcher, Sandberg et al. 1987; Uematsu, Greenberg et al. 1989; Auer 2004; Suh, Hamby et al. 2007).

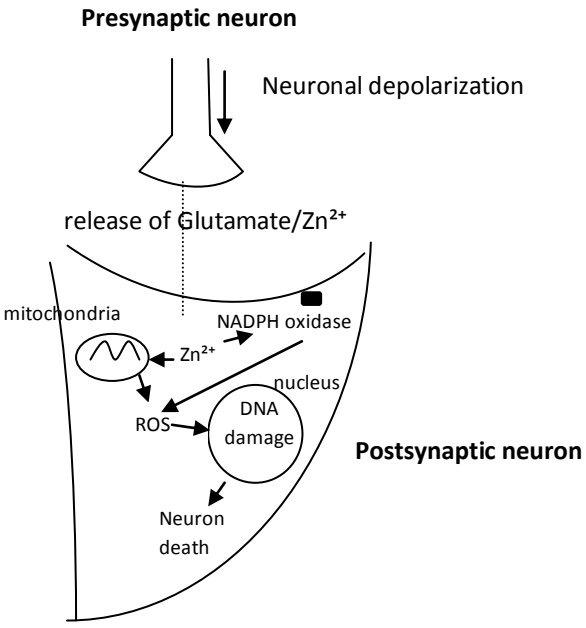

Figure 18. Hypoglycemia-induced neuronal death.

It is important to consider the difference between the adult and newborn brain reactions to hypoglycemia and the relative resistance of the immature brain to hypoglycemic brain damage (Yager 2002) which is attributed to:

- Enhanced CBF and cerebral uptake of glucose
- Enhanced ability to use alternate substrates (especially lactate)
- Decreased requirement for glucose utilization
- Preservation of cerebral high-energy phosphates

In the newborn, despite the preservation of cerebral energy status even in severe hypoglycemia (<1 mmol/l), the possible underlying cause of cellular injury may be related to the release of EAAs (Yager 2002). Studies have shown an increase in EAA concentrations due to neonatal hypoglycemia (Silverstein, Simpson et al. 1990; Aral, Gucuyener et al.

1998). Insulin-induced hypoglycemic seven-day-old rat pups showed increased concentrations of extracellular glutamate with lower levels of blood glucose (Silverstein, Simpson et al. 1990). Moreover, increased levels of aspartate and glutamate were reported in the CSF of hypoglycemic newborn human infants in a study done by Aral et al. (Aral, Gucuyener et al. 1998).

Hypoglycemia may cause a drop in electrical activity of the brain, membrane breakdown with release of free fatty acids, and altered amino acid metabolism, including increased production of glutamate. Excitatory amino acid neurotransmitters are believed to play a major role in the hypoglycemic-induced brain injury through overactivation of NMDA receptors (McGowan 1999). Even though these neurotransmitters have critical roles during early brain development, studies on rats indicate that immature brain may be susceptible to disproportionate amounts of glutamate, aspartate, glutamine, and GABA (Jantzie, Rauw et al. 2006). NMDA receptors predominate in immature brain including human and rat, and are associated with an ion channel that transports sodium and calcium into the cell and potassium out of cell. There is an increased number of these ionotropic glutamate receptors in the late fetal and early newborn periods which is believed to be involved in formation of synaptic connections and arborization of dendrites (synaptogenesis) and programmed cell death (apoptosis). In addition, NMDA receptors in the immature brain appears to be less sensitive to the magnesium block than

the adult brain (Puka-Sundvall, Gilland et al. 1996). Even though normal activity of NMDA receptors is critical to the normal brain development, their overactivation by excessive concentrations of synaptic EAA neurotransmitters causes neuronal homeostatic instability and alteration of transmembrane ion gradients via increase in cytoplasmic levels of sodium and calcium. Hypoglycemia increases the sensitivity of NMDA receptors to activation by glutamate, which may result in a lower threshold for glutamate-induced excititoxicity (McGowan 1999). During hypoglycemia energy-dependent mechanisms for restoring normal transmembrane gradients of ions are impaired due to depletion of ATP and phosphocreatine. Excess calcium influx activates cellular enzymes such as phospholipases and proteases, deregulates mitochondrial metabolism, alters patterns of synaptic transmission, induces production of ROS, damages DNA, and eventually may result in selective neuronal necrosis (McGowan 1999; Suh, Hamby et al. 2007).

Several studies have revealed that endogenous carbohydrate and amino acid stores are used as substrates in hypoglycemia. Hypoglycemia causes a rise in tissue aspartate as a result of the aspartate aminotransferase reaction: glutamate + oxaloacetate ↔ aspartate + α-ketoglutarate, and a drop in glutamate, while both amino acids flood the extracellular space of the brain. Release of extreme amounts of glutamate into synaptic cleft, coupled with an impaired astrocytic glutamate reuptake mechanism due to hypoglycemia causes increased

extracellular glutamate. Other amino acids such as glutamine, GABA, and alanine have also been shown to be reduced in the brain tissue during severe insulin-induced hypoglycemia. Inhibitory amino acids similarly may flood the extracellular space; however, their inhibitory effects are not sufficient to prevent hypoglycemic convulsions in the face of the excitatory amino acid release (Norberg and Siesio 1976; Sandberg, Butcher et al. 1986; Auer 2004).

The significant reduction in tissue concentrations of glutamine and alanine along with an increase in aspartate found in our study mimics findings in the literature. Decreased tissue glutamine and alanine likely reflect higher utilization of these amino acids for energy metabolism. On the other hand, this may also be caused by inhibition of synthesis due to depletion of energy stores during hypoglycemia (Sandberg, Butcher et al. 1986). Since alanine is generated by the transamination of pyruvate from glycolysis, decreased alanine may be indicative of decreased glycolysis after hypoglycemia (Jantzie, Rauw et al. 2006). We speculate that the lower levels of intracellular glutamine and alanine in hypoglycemia may lead to their less release during severe ATP depletion. In addition, glutamine is hydrolyzed to glutamate in the neurons to replenish the glutamate that neurons release to the synapse (Yudkoff, Daikhin et al. 2008). Increased tissue aspartate is believed to result from transamination and the conversion of tissue glutamate to aspartate, as suggested above.

Glutamate did not show a significant decrease in the brain tissue of our hypoglycemic animals in contrast to other studies. This might be due to the severity and duration of hypoglycemia that we induced in our experiments. We speculate that under such severe-prolonged hypoglycemic conditions (≤1.1 mmol/L for ≥12 hr), the aspartate-glutamate transaminase reaction – which causes the continuation of Krebs cycle in the absence of glucose (Figure 19) – may not be the preferred pathway for ATP generation and other pathways by using other substrates such as lactate and ketone bodies might take priority under these conditions.

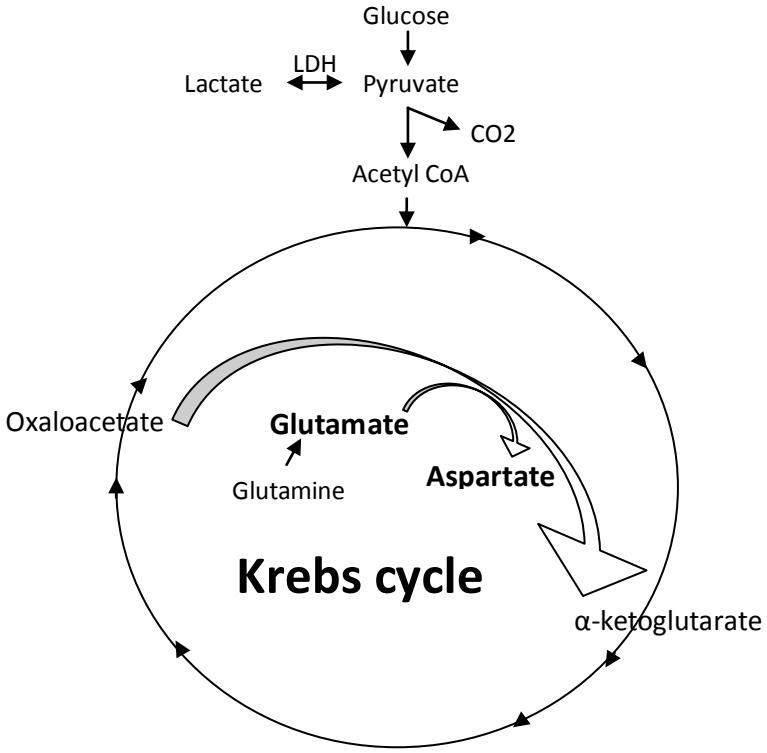

Figure 19. Krebs cycle in hypoglycemia is truncated - Hypoglycemia causes a rise in tissue aspartate as a result of the aspartate aminotransferase reaction (glutamate + oxaloacetate ↔ aspartate + α-ketoglutarate).

As mentioned, other studies have also shown decreased GABA in brain tissue due to hypoglycemia (Sandberg, Butcher et al. 1986). The inconsistency between this and our results may be explained by the difference between GABA precursor concentrations in two studies. Glutamate is the precursor for GABA production (Madl and Royer 2000) and because there was no reduction in glutamate in our severely hypoglycemic brains, no GABA decrease was therefore found in our samples.

Since arginine is the precursor for nitric oxide (NO) - a reactive oxygen intermediate (free radical) - the increased concentrations of cerebral arginine, particularly in thalamus, might hypothetically result in increased levels of nitric oxide and explain the early brain injury in TRN of the severely hypoglycemic pups in our study. In our hypoglycemic animals, the TRN vulnerability might be as a result of high metabolic demands of this region of the rat brain during early developmental stage and therefore more sensitivity to energy depletion followed by release of extreme amounts of excitatory amino acids (excitotoxicity) and the subsequent events (described previously) resulting in neuronal cell death. In vivo microdialysis technique (Sandberg, Butcher et al. 1986) that can provide a better indication of extracellular excitatory and inhibitory amino acid levels may shed some light on this, and allow the determination of whether excitotoxicity is involved in the damage seen in TRN.

Reactive Oxygen Species (ROS), Oxidative Stress, and Matrix Metalloproteinases (MMP)

We analyzed MMP-2 concentrations and the brain redox ratio, as an indicator of oxidative stress, in the immature rat brain. Higher concentrations of MMP-2 in the cortex, hippocampus, and thalamus in our study may indicate higher oxidative stress in the brain tissue of the long-severe hypoglycemic rats. In addition, even though Redox Ratio did not show significant increase in each evaluated areas of our hypoglycemic brains specifically, it was increased in the whole brain and therefore may reflect increased oxidative damage as a result of hypoglycemia.

The immature brain is highly vulnerable to oxidative damage due to a high rate of oxygen consumption, high concentrations of unsaturated fatty acids, low concentrations of protective antioxidants such as glutathione peroxidase, and the availability of redox-active iron (Ferriero 2004). There is increasing evidence that the ability of mitochondria to produce ROS is increased after acute hypoglycemia in the immature brain. Increased ROS may result in changes in brain structure and function through oxidant injury to mitochondrial proteins and DNA or alterations in oxidant-sensitive signal transduction pathways in the brain (McGowan, Chen et al. 2006).

During feto-neonatal development, MMPs play an important role in tissue growth and morphogenesis through their ability to degrade all kinds of extracellular matrix proteins (ECM) such as collagens, laminin, gelatin,

elastin, fibronectin, and proteoglycans. These zinc-containing endopeptidases are involved in connective tissue remodeling in both normal physiologic conditions such as wound healing, tissue development, bone growth, angiogenesis and pathologic processes including inflammatory diseases, tumor invasion and metastasis, atherosclerosis, central nervous system diseases (Woessner 1991; Jiang, Namura et al. 2001; Schulz, Sawicki et al. 2004). Studies have also shown a role of MMPs in the pathogenesis of brain damage resulting from cerebrovascular disease (Mun-Bryce and Rosenberg 1998). MMP-2 (gelatinase A:pro-enzyme, 72 kDa), which is involved in acute disease processes such as platelet aggregation (Sawicki, Salas et al. 1997), myocardial dysfunction after ischemic-reperfusion (Cheung, Sawicki et al. 2000), and the regulation of vascular tone (Fernandez-Patron, Radomski et al. 1999), is activated through proteolytic cleavage and/or oxidative stress (Okamoto, Akaike et al. 1997). MMP-2 degrades the major components of basal lamina around cerebral blood vessels (type IV collagen, laminin, and fibronectin) (Lukes, Mun-Bryce et al. 1999). Post-ischemic activation of MMP-2 has also been reported in both experimental animals (Jiang, Namura et al. 2001) and human patients (Clark, Krekoski et al. 1997). Failure of newborns to adapt extrauterine environmental conditions such as enhanced oxidative stress, as may occur in preterm and critically ill neonates may develop a variety of disorders such as bronchopulmonary dysplasia (BPD) and intraventricular hemorrhage

(IVH). MMPs may be involved in the pathophysiological processes of these disorders via their actions on the remodeling of the ECM, platelet aggregation, and vasomotor regulation (Saugstad 1988; Rogers, Witz et al. 2000). Because MMPs are activated by proteolysis and oxidants (Stevens, Churchill et al. 2008), the significantly increased concentrations of MMP-2 in the cortex, hippocampus, and thalamus in our study may indicate higher oxidative stress in the brain tissue of the long-severe hypoglycemic rats. Increased MMP-2 activity could also be implicated in the cellular damage seen in the TRN.

Redox ratio, which is the tissue marker of oxidative stress, was calculated as the ratio of reduced glutathione, determined by subtracting oxidized glutathione (GSSG) from total glutathione (GSH) levels, to GSSG (Johnson, Bigam et al. 2007). Glutathione is an endogenous antioxidant that can provide neuroprotective effects and protects cells from toxins such as free radicals (Wang, Cheng et al. 2003). Glutathione is found almost exclusively in its reduced form, since the enzyme which reverts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used scientifically as a measure of cellular toxicity (Pastore, Piemonte et al. 2001). As mentioned before, this ratio even though did not show significant increase in the cortex, hippocampus and thalamus of our hypoglycemic brains specifically, its increase in the

whole brain tissue may reflect increased oxidative damage as a result of hypoglycemia.

It might be questioned that if increased EAA concentrations and/or MMP-2 activity could cause brain damage in the thalamus (TRN), why it did not cause injury in other regions of the brain (cortex and hippocampus). This may be due to involvement of other factors in brain injury other than or in parallel with EAAs and MMPs in different regions of brain. It is entirely possible that these areas of the brain are exposed to metabolic alterations that place them on the threshold of injury, but are not significant enough to cause damage, perhaps emphasizing the role of energy supply vs. demand in different regions of the developing brain. Also, it is unknown what concentrations of EAA or MMP can cause brain injury in different areas of the brain. Therefore, a given level of EAA or MMP might result in damage in a specific region of the brain while the same concentration of the same amino acid or enzyme cannot affect other regions.

Conclusions and Future Directions

In conclusion we did not observe any effect of isolated hypoglycemia on overall behavioral outcomes. Early pathologic alterations (PD9) were detected only in those animals exposed to severe prolonged hypoglycemia, AND in a region specific manner (TRN). Even so, the pathologic alterations were not sustained. Severe prolonged

hypoglycemia also resulted in early metabolic alterations of excitatory amino acids and matrix metalloproteinases in a more diffuse grey matter pattern.

Hypoglycemia is a common event in newborn infants, and our data provides new information that suggests that despite the metabolic and early pathologic alterations occurring in the hypoglycemic brain, neonatal hypoglycemia did not cause overt *permanent* brain damage in the immature rat. This might indicate the relative resistance of the immature rat brain to insulin-induced neuronal injury during the early postnatal period. The mechanisms through which such neural protection occurs is still unknown and require further studies.

In addition, further research would be required to determine whether the early metabolic alterations and transient neuronal injury results in *subtle* long term behavioral and/or pathologic abnormalities. This can be assessed by performing longer term and more specific behavioral tests in addition to looking at more detailed specifications of neuronal structures such as synaptogenesis, thickness, length and branching of axons and dendrites by using synaptophysin or golgi staining.

Hypoglycemia is often not an isolated phenomenon in newborn infants and is frequently combined with either HI or seizures particularly in premature newborns. Therefore, in a discussion of human neonatal hypoglycemic brain damage, concurrent perturbations should also be

considered. Studies have indicated the aggravation of brain injury when different insults are combined (Wirrell, Armstrong et al. 2001; Yager, Armstrong et al. 2002). Therefore, it would be worthwhile to perform a similar series of experiments to those performed in this thesis in which an additional group of animals were exposed to a 'mild' hypoxic-ischemic insult as well as being rendered hypoglycemic. This will allow determining the effect of combined hypoglycemia and asphyxia on neuropathologic and behavioral outcome in an animal model reflective of the near term asphyxiated baby.

From a clinically relevant perspective, it appears that severe hypoglycemia places the immature brain 'at risk' for injury, even though damage may not be permanent, as in our experiment. However, given the findings of our research regarding transient histo- biochemical alterations we would recommend early diagnosis and treatment of the newborn with hypoglycemia to avoid severe hypoglycemia. This particularly may be true under clinical circumstances that may further compromise the supply vs. demand equilibrium, leading to neuronal injury.

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