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THE ROLE OF EXCITATORY AMINO ACIDS AND LACTIC ACIDOSIS IN HYPOGLYCEMIC BRAIN DAMAGE

by

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science.

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The undersigned certify that they have read, and recommended to the Faculty of Graduate studies for acceptance, a thesis entitled, "The Role of Excitatory Amino Acids and Lactic Acidosis in Hypoglycemic Brain Damage" submitted by Marios Papagapiou in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Hypoglycemia is a systemic insult in which the heart continues to beat but brain function, evidenced by electrical activity demonstrable in the electroencephalogram (EEG), fails. Histological investigation reveals that the major type of brain damage incurred after profound hypoglycemia is selective neuronal necrosis (SNN), defined as death of neurons with sparing of the glia. There is a positive correlation between the duration of EEG isoelectricity (total absence of spontaneous EEG activity) and the number of necrotic neurons observed.

The histologic aberrations observed in hypoglycemic brain damage are thought to be mediated by the N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor, supported by the fact that intrastriatal injections of the NMDA antagonist 2-amino-7-phosphonoheptanoic acid (AP7) prior to isoelectricity protect the striatum from hypoglycemic neuronal necrosis.

The most potent non-competitive NMDA receptor antagonist is (+)-5-methyl-10,11-dihydro-5-H-di(a,d)cyclohepten-5,10-imine or (MK-801). This compound readily crosses the blood brain barrier, and was tested for its effect in mitigating hypoglycemic brain damage. In the first project of this thesis, regimens of 3 different doses of MK-801, injected intravenously (iv) during the stage of delta EEG activity, histologically protected the striatum, hippocampus and cortex in

animals which underwent 30 min of isoelectricity and 7 days recovery.

In the second project of this thesis, quinolinic acid (QUIN), a tryptophan metabolite was studied. Levels of this endogenous neurotoxin, lethal to neurons in nanomolar quantities, were measured in brain and plasma. Male Wistar rats with 40 min of EEG isoelectricity and 1 h of subsequent normoglycemic recovery were found to have elevated QUIN concentrations in whole brain homogenates. Subsequent intracebral microdialysis showed no increase in QUIN, but plasma QUIN levels were markedly increased, suggesting that the increase in brain QUIN was attributable to an increase in blood QUIN contained in the brain samples. The results provide strong evidence against a role of QUIN in hypoglycemic neuronal necrosis.

In the third project of this thesis, manipulation of recovery blood pressure and blood glucose showed that profound hypotension (35-40 mm Hg) in conjunction with hyperglycemia (>15 mM) exacerbates cerebral lactate accumulation and can lead to brain infarcts. During profound hypoglycemia, lactic acid production is curtailed by lack of glucose. However upon recovery with glucose, lactate production increases. The results suggest that hypotension and hyperglycemia should be avoided clinically in the recovery period after hypoglycemia, in order to avoid lactate-mediated brain infarcts.

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Also I would like to express my profound appreciation and gratitude to my parents, Andreas and Evangelia, and to my sister Katina for their love and continued support for my

family, and for my academic aspirations. Thank you all.

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LIST OF ABBREVIATIONS

Ala	Alanine
ANOVA	Analysis of Variance
AP7	2-amino-7-phosphonoheptanoic Acid
Asp	Aspartate
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BP	Blood Pressure
°C	Degrees Celsius
Ca^{2+}	Calcium ion
Ca^{2+}_e	Extracellular calcium concentration
CBF	Cerebral Blood Flow
Cl^-	Chloride ion
cm	centimeter(s)
CMR_{glu}	Cerebral Metabolic Rate for Glucose
$CMRO_2$	Cerebral Metabolic Rate for Oxygen
CNS	Central Nervous System
CoA	Coenzyme A
CSF	Cerebrospinal Fluid
CSS	Control Salt Solution
DA	Dopamine
AP5	2-amino-5-phosphonopentanoic acid
d.c.	Direct current

KA	Kainic Acid
K^+_e	Extracellular potassium concentration
l	liter
LCBF	Local Cerebral Blood Flow
$LCMR_{glu}$	Local Cerebral Metabolic Rate for Glucose
LDH	Lactic Acid Dehydrogenase
L-glu	L-glutamate
L-TRP	L-Tryptophan
M	Molar
min	Minute(s)
mm	millimeter(s)
mM	millimole(s)
MK-801	(+)-5-methyl-10,11-dihydro-5-H-(a,d)cyclohepten-5,10-imine
mV	millivolts
Na^+	Sodium ion
NAD	Nicotinamide Adenine Dinucleotide (Oxidized form)
NADH	Nicotinamide Adenine Dinucleotide (Reduced Form)
NE	Norepinephrine
NMDA	N-methyl-D-Aspartate
nmol	nanomole(s)
µl	microliter(s)
µmol	micromole(s)
OAA	Oxaloacetate
DOPAC	3,4-dihydroxyphenylacetic Acid

p_aCO_2	Partial pressure of carbon dioxide (arterial blood)
p_aO_2	Partial pressure of oxygen (arterial blood)
PCr	Phosphocreatine
3-PGDH	Phosphoglyceraldehyde-3-phosphate dehydrogenase
pmol	picomole(s)
Q	Quisqualic Acid
QUIN	Quinolinic Acid
[^{3}H]-QUIN	Tritiated Quinolinic Acid
RNA	Ribonucleic Acid
RER	Rough Endoplasmic Reticulum
s	second(s)
SNN	Selective Neuronal Necrosis
xg	Times gravity (9.8 m/sec^2)

DEDICATION

I would like to dedicate this thesis to my wife Panayiota, for her continued love, support and unqualified patience, and to our sons, Andreas and Christiphoros who have given us unbound joy and happiness.

INTRODUCTION

With the discovery of insulin in 1921 a new era of treatment was ushered in for the dreaded disease diabetes mellitus. Insulin is a two chain (α and β) 51 amino acid residue protein with one intra, and two inter chain cysteine disulfide linkages. The early use of insulin was not restricted to diabetics. Insulin was also used iatrogenically in the treatment of schizophrenia and other psychiatric disorders. The pharmacological dosages administered for psychiatric disorders resulted in insulin coma, the desired therapeutic duration of which was 30 min. The results obtained from this treatment modality were thought by some early researchers to be the effect of insulin itself, rather than hypoglycemia. (See Auer [1986] for a review).

Conditions of either oxygen or glucose deprivation would seem at the outset to be similarly detrimental to brain cells [Auer and Siesjö 1988] thus a deficiency of either of these might be thought sufficient to cause damage to metabolically active cells. The tenet that substrate (glucose) starvation of neurons is simply and directly responsible for the hypoglycemic neuronal necrosis was already challenged in the thirties. As early as 1938 the American neuropathologist Arthur Weil, experimenting with rabbits and hyperinsulism observed that the damage in the hippocampus could be more

pronounced in structures which are in apposition to the cerebral ventricles, specifically in the dentate gyrus [Weil et al 1938]. This histological observation was critical in Weil's proposal that perhaps the damage is related to a cerebrospinal (CSF) borne toxin. Further detailed studies by Auer and associates have shown that the distribution of hypoglycemic brain damage in the rat dentate gyrus shows a relationship to the subarachnoid cisterns, the fluid spaces around the brain [Auer et al 1984b]. After 1 week survival, the damage of the dentate gyrus was localized to neurons located beneath the subarachnoid cistern above the third ventricle. Injections of the protein tracer horseradish peroxidase into the CSF of normal rats selectively labels the crest of the dentate gyrus [Erzurumlu et al 1981]. The subsequent transport of the protein to the neuronal perikarya reveals labelling of cells of the dentate having their dendrites oriented toward the cisterns, with the possibility of direct contact with the CSF. The labelling is virtually identical to the pattern of damage in the dentate gyrus after hypoglycemic isoelectricity. These observations lend support to the hypothesis of a fluid borne toxin.

The evolution of necrosis in hypoglycemic brain damage shows very specific features. A dendrosomal axon-sparing lesion under the electron microscope begins with dendritic swelling, followed by mitochondrial swelling, and lastly

dendritic cell membrane breaks. Finally, pathologic alterations and eventual necrosis of the neuronal perikarya are observed [Auer et al 1985a].

There is a positive correlation between the duration of EEG isoelectricity (total absence of spontaneous EEG activity) and the number of necrotic neurons observed. As blood glucose levels fall, the EEG progressively declines in amplitude, and decreases in frequency from normal (8-13 Hz) to the theta range (4-8 Hz) and finally to the slowest range of frequencies, the delta range (1-4 Hz). Further derangement of EEG leads to the burst suppression pattern, where large delta wave spikes alternate with total suppression of electrical activity [Auer et al 1984a].

The general thinking in the early days of brain research in hypoglycemia was that in conditions of either oxygen or (substrate) glucose deprivation, the cells finally succumb due to internal energy store depletions and aberrations in the neurochemical homeostasis. The vulnerability of neurons in relation to glia was simply explained due to their active metabolism. Therefore hypoglycemia was considered to be a form of ischemia [Courville 1957, Brierley et al 1973]. This general view prevailed until the advent of techniques for the measurement of Cerebral Blood Flow (CBF), cerebral metabolic rate for glucose (CMR_{glu}) and neurochemical parameters, which made differences between ischemic and hypoglycemic brain

damage readily apparent. With the accumulation of neurochemical data it became evident that hypoglycemia and ischemia were indeed two distinct brain pathologies. For example, neurochemical whole-brain analyses revealed in ischemia that cellular redox systems were reduced [Rehncrona et al 1980], while in hypoglycemia were oxidized [Norberg and Siesjö 1976]. In hypoglycemia, during the period of isoelectricity, brain pH shifts toward alkalinity [Pelligrino and Siesjö 1981]. This alkalosis is due to intracellular deamination of proteins [Lewis et al 1974] producing the strong base, ammonia, and to the oxidation of metabolic acids [Agardh et al 1980]. In ischemia there is an increase in brain pH due to an excess synthesis of lactic acid and proton production [Rehncrona et al 1981, Pulsinelli et al 1982].

Although the brain normally utilizes oxygen and glucose in a stoichiometric relationship, in hypoglycemia, depression of cerebral oxygen consumption is less than expected from theoretical predictions [Pappenheimer and Setchell 1973, Agardh et al 1981a]. This indicates that the brain, in an emergency situation, where its substrate for oxidative metabolism is diminished or restricted, is in a position to utilize other endogenous molecules for its required energy needs. In ischemia the levels of ATP concentrations are reduced to approximately 5% of control, while in hypoglycemia the reduction of the high energy phosphate molecules is

reduced between 25 and 30% of control. This large difference in ATP concentrations is due to the fact that during isoelectricity in hypoglycemia, the brain is oxidizing other endogenous molecules, while during ischemic isoelectricity this is not the case.

The histopathology observed in hypoglycemia and ischemia is also distinct in its distribution and severity. In hypoglycemia, the most vulnerable regions of the brain which are susceptible to hypoglycemic brain damage are the hippocampus (especially the dentate crest and gyrus), caudate nucleus, and the cerebral cortex [Auer et al 1984b, Auer et al 1985a, Kalimo et al 1980, Kalimo et al 1985]. In ischemia, the damage is restricted to the CA1 band of the hippocampus, cerebral cortex and the caudate nucleus. Hypoglycemic isoelectricity can be maintained up to an hour with mortality approaching approximately 50%, while in ischemia, conditions of ischemic blood blow in rats cannot be maintained longer than 10-12 min with any survival. In a pure hypoglycemic insult the brain pathology observed is that of SNN, pan-necrosis is never observed. In ischemia, both SNN and pan-necrosis are observed. There is correlative evidence that pan-necrosis in some pathologic conditions is related to a build up of tissue lactic acid [Hakim 1984, Auer et al 1986b, Kuriyama et al 1984, Ingvar et al 1987].

Quantitative autoradiographic studies by several groups

have shown that a large percentage (>80%) of the glutamate binding sites in the vulnerable CA1 zone of the hippocampus are of the NMDA subtype [Monaghan et al 1983, Greenamyre et al 1985]. There is thus a good correlation between the distribution of observed brain damage in hypoglycemia and the distribution of NMDA receptors.

The experiments in this thesis were designed to basically attempt to answer three questions relating to hypoglycemic brain damage. In the first project, the hypothesis was tested that blockade of the NMDA receptor by the selective and potent non-competitive antagonist, MK-801 [Wong et al 1986], given parenterally prior to hypoglycemic isoelectricity would mitigate hypoglycemic brain damage. The second project was designed to address the question of whether QUIN concentrations in the brain would increase during profound hypoglycemia, since QUIN is synthesized in the brain [Okuno et al 1987], is a powerful neurotoxin [Schwarcz et al 1983] and since insulin increases tryptophan uptake in the brain [Wurtman et al 1981]. The third project of the thesis tested the hypothesis that severe hypotension in conjunction with hyperglycemia would cause lactic acidosis and infarction in addition to classic hypoglycemic SNN. A background outlining the neurochemistry, blood flow and pathology of hypoglycemic brain damage will now be given.

Neurochemistry

During hypoglycemic isoelectricity, lack of glucose for energy metabolism causes the brain to shift from glucose to endogenous brain substrates as sources of energy [Abood and Geiger 1955]. The initial 5 min of hypoglycemic coma induces an 8 to 10% decrease in phospholipid content in brain tissue, while there is a concomitant increase in the free fatty acid (FFA) content [Agardh et al 1981a]. It has been speculated that the phospholipids which are oxidized during hypoglycemia are scavenged from membranes of internal organelles, particularly those of the endoplasmic reticulum and mitochondria [Agardh et al 1982]. Perturbation of cerebral metabolism of catechol and indole amines is also observed during severe hypoglycemia. Throughout isoelectricity, concentrations of dopamine (DA), norepinephrine (NE) and 5-hydroxytryptamine (5-HT) are reduced, while the concentration of 5-hydroxyindole acetic acid (5-HIAA) is increased, suggesting an increased catabolism of these biogenic amines [Agardh et al 1979]. Utilization of proteins and ribonucleic acid (RNA) is also observed during severe hypoglycemia [Agardh et al 1981a]. Protein synthesis in the brain during and following isoelectricity is differentially suppressed and recovery is time-dependent. The vulnerable brain regions which have been delineated during severe hypoglycemia (cerebral cortex,

hippocampus, caudoputamen) regain their protein synthetic capacity within 3 to 6 h after recovery from hypoglycemia. Neurons of the CA1 sector of the hippocampus, and small neurons of the caudoputamen have a delay in the resumption of their protein synthesizing capabilities well beyond 6 h recovery. In the caudoputamen, necrosis of some cells is reflected by absence of the capacity to synthesize proteins well after 7 days of recovery [Kiessling et al 1982, Kiessling et al 1984, Kiessling et al 1986]. Within the first 5 to 10 minutes of isoelectric coma, amino acid concentrations plateau in whole tissue homogenates. This leveling off indicates that substrate utilization by transamination or deamination has achieved a new level of homeostasis within 5 min of isoelectricity [Norberg and Siesjö 1976, Agardh et al 1978]. The energy charge (e.c.=ATP+½ADP/ΣATP+ADP+AMP) [Atkinson 1968], defined as the extent to which the ATP-ADP-AMP system is "filled" with high energy phosphate groups of the cells, is also blood pressure-dependent. During isoelectricity the cortical e.c. is approximately 0.58 at a blood pressure of 140 mm Hg, 0.53 at 100 mm Hg, and it is further decreased to 0.37 when the blood pressure is allowed to fall to 80 mm Hg. At pressures of 100 and 80 mm Hg, phosphocreatine (PCr) and ATP concentrations are reduced to 5 and 10% of control respectively [Auer et al 1986a]. The cerebellum's ability to function under isoelectric duress is more robust than other

brain structures. Deterioration of the e.c. in the cerebellum is less pronounced than in the cerebral cortex, and the FFAs are detected later in the cerebellum than in the cerebral cortex. In fact these moities are increased in the cortex within 5 to 10 min of cerebral isoelectricity, but no increase in cerebellar tissue can be detected until aproximately 1 h of isoelectricity has elapsed. Glycogen stores are depleted in the cerebral cortex within 5 min of isoelectricity, but in the cerebellum, concentrations greater than 0.1 μmol/g are still detectable. In the cerebellum, cellular levels of pyruvate and lactate are maintained, suggesting that there is still active glycolysis in this region during hypoglycemic isoelectricity [Ratcheson et al 1981, Agardh and Siesjö 1981].

During severe hypoglycemia, perturbations in the neurochemistry can theoretically take place in either intracellular or extracellular loci. In terms of specific amino acids, there is a reduction in the whole tissue concentration of glutamate (glu), glutamine (gln) gamma-amino butyric acid (GABA) and alanine (ala) [Norberg and Siesjö 1976], while tissue aspartate (asp) is dramatically elevated, likely due to the increased transamination of glu and oxaloacetate (OAA). Due to lack of glucose during isoelectricity, there is also a decline in the concentrations of pyruvate. This fall in pyruvate might be responsible for the depletion of citric

acid cycle substrates. A decrease in pyruvate will cause decreased availability of acetyl CoA, thus condensation with OAA to form citrate is diminished. This will drive the asp amino-transferase reaction to the right:

glutamate + OAA $\rightleftharpoons$ aspartate + α-ketoglutarate

This reaction, which provides amino acid carbon skeletons into the citric acid cycle, would explain a decrease in glu and a subsequent rise in asp within cells [Lewis et al 1974]. The extracellular concentration of asp during hypoglycemic isoelectricity increases approximately fifteen fold above baseline (in vivo hippocampal dialysis) while glu increases five fold [Sandberg et al 1986]. Within 30 min of glucose induced recovery, levels of the neuroactive amino acids (asp, glu, GABA) are restored to normal or near normal values.

Since the tissue energy stores during isoelectricity are progressively depleted, cells cannot maintain the high intra to extracellular gradient (approximately 1000 fold for Asp [Sandberg et al 1986]) of these acidic amino acids; thus the energy-requiring, high-affinity uptake process of these amino acids is compromised and amino acids leak out into the extracellular space. Ionic derangement during severe hypoglycemia has a marked effect on potassium and calcium homeostasis. Using microelectrodes to measure extracellular potassium (K^{+}_{e}) and calcium (Ca^{2+}_{e}), several researchers have identified a biphasic derangement in the homeostasis of these ca-

tions. Astrup and Norberg have found no correlation between the onset of isoelectricity and release of intracellular potassium [Astrup and Norberg 1976]. However, the initial extracellular concentrations (K^+_e and Ca^{2+}_e) of these cations measured with sensitive ion-selective microelectrodes are 3.4 ± 0.94 and 1.17 ± 0.14 mM respectively [Harris et al 1984]. A depolarization is observed during the burst suppression pattern or during the onset of isoelectricity. This depolarization resembles a cortical spreading depression. During the first depolarization there is an increase in the K^+_e (13 mM) and with a delay of 7.8 ± 1.4 s, there is a concomitant decrease in Ca^{2+}_e (peak K^+_e and Ca^{2+}_e 48 and 0.18 mM respectively [Harris et al 1984]). After the first depolarization, a second one occurs with a time lag of approximately 1.5 min. The amplitude of the second depolarization is, in essence a mirror image of the first. Measurements of cortical tissue adenylate species indicate that their decline does not occur until a spreading depression is elicited [Harris et al 1984, Wieloch et al 1984]. A decrease in ATP concentrations would have a deleterious effect on the function of ATPase. Decreased ATPase function would lead to a prolonged membrane depolarization, and with time this depolarization would induce a cortical direct current (d.c.) potential of 0 volts [Astrup et al 1981, Hansen and Zeuthen 1981].

Cerebral Blood Flow and Metabolism

Hypoglycemia reduces cerebral glucose metabolism due to the fact that substrate availability is curtailed, but the cerebral metabolic rate for O_2 ($CMRO_2$) is barely compromised. This is due to the brain's ability to oxidize endogenous substrates for its energy requirements when glucose availability is diminished. Ghajar and his associates showed that during hypoglycemic stupor, the electrographic counterpart of which is the delta stage of EEG activity, cerebral ATP declined by 6% from control, while during isoelectricity ATP declined by 40% and $CMRO_2$ by 45% and CMR_{glu} by 73% respectively [Ghajar et al 1982].

During the period of isoelectricity cerebral autoregulation is lost [Abdul-Rahman et al 1980], and local cerebral blood flow (LCBF) is differentially affected. LCBF increases on the average approximately 2.5 fold over control values; to 1.4 times control in the hippocampus and to 3.2 times control in the cerebellum [Abdul-Rahman et al 1980]. Upon recovery from isoelectric coma the LCBF is decreased in the brain (45-60% of control values) except in the cerebellum, where it remains above control values. Arterial plasma glucose concentrations seem to be the dominant factor determining glucose availability [Abdul-Rahman and Siesjö 1980]. Bryan and his associates have corroborated Abdul-Rahman's results in

unanesthetized hypoglycemic rats [Bryan et al 1987]. Using iodoacetic acid (IAA), a specific inhibitor of the glycolytic enzyme phosphoglyceraldehyde-3-phosphate dehydrogenase (3-PGDH), thus simulating hypoglycemia, Tanaka was able to show an intact LCBF but a reduction in local cerebral glucose metabolism ($LCMR_{glu}$) [Tanaka et al 1985]. Oxygen availability was intact, but pyruvate production was indirectly inhibited due to the inhibition of 3-PGDH.

Neuropathology

The histopathology of hypoglycemic brain damage has several distinct hallmarks. During early isoelectricity the damage observed is characterized as Type I injury. By light microscopy, damaged neurons have an angulated perimeter, are darkly stained with perineuronal vacuolations and the affectation is generally restricted to small neurons of the third cortical laminae. Type II injury is characterized by swollen neurons, with some vacuolation of the peripheral cytoplasm, but with no distinct nuclear changes. This injury is generally restricted to the deeper cortical laminae, such as 5 and 6. The severity of the damage is relatively graded, from superficial to deep cortical laminae, the most severe being at the superficial layers [Agardh et al 1980]. Type II neuronal damage is considered to be reversible. Studies carried

out utilizing electron microscopy have confirmed the studies at the light microscopic level. Type I cell injury is characterized by cellular shrinkage, darkly stained cytoplasm, compact ribosomes, elongation of the mitochondria and dilation of the rough endoplasmic reticulum (RER) and the Golgi cisternae. Type II injury is more subtle. Apart from the neuronal swelling, the mitochondria are contracted, ribosomes are disintegrated and the RER seems to be reduced in volume [Kalimo et al 1980].

With the advent of a recovery model for hypoglycemic brain damage, the distribution and time course of the damage was delineated in greater detail. Work carried out by Auer and his associates extended earlier investigators' histologic observations. In the cerebral cortex of the rat, the neuronal necrosis seen at 7 days recovery was graded, the severity of damage being greatest in the superficial layers (laminae 2-3) and decreased into the deeper layers [Auer et al 1985a]. Dark and light neurons, corresponding to the above-described type I and type II injury respectively began to appear in the cerebral cortex as early as 10 min of isoelectricity. In laminae 4-6 dark cell damage was reversible within 24 h of recovery. Dark neurons, characterized by hyperchromasia [Auer et al 1985a] transformed into acidophilic neurons by 6 to 8 hours.

Acidophilic neurons are characterized by their affinity

for acidic dyes such as eosin, acid fuchsin or phloxine. Microscopically, these cells show extensive, irreversible damage to their mitochondria in the form of flocculent densities, and rupture of the cell and nuclear membranes (cytorrhexis and karyorrhexis respectively). In addition, they fragment and disappear from the tissue (cytoclasis) with the passage of time. Together, these features establish acidophilic neurons as necrotic [Auer et al 1985a, Auer et al 1985b].

The hippocampus is damaged in the subiculum, dentate gyrus and CA1 band. The external blade of the dentate, along with the CA3 band are consistently damaged if the period of isoelectricity is increased to 45 to 60 min [Auer et al 1984a, Auer et al 1985b]. The striatum is also affected during electrocerebral silence. Upon restitution to normoglycemia, damage is observed within 7 min. The damage is accentuated near the white matter. In more severely affected animals the SNN can extend near the angle of the lateral ventricle [Auer et al 1984a, Kalimo et al 1985]. The cerebellum was usually spared during isoelectricity. Damage was seen when the duration of isoelectricity was extended approximately 45 to 60 min especially in cells that were located immediately in the vicinity of the foramen of Luschka [Agardh and Siesjö 1981, Auer et al 1984a]. Again, this information lends support to Weil's hypothesized fluid borne toxin.

The NMDA Receptor and Neurotoxicity

Selective neuronal necrosis is a distinct form of pathology seen in association with the hyperexcitation of neurons by an excitatory amino acid (EAA) neurotransmitter(s). Hyperexcitation can be defined as a state of overt or continuous stimulation of a neuron [McLennan and Lodge 1979]. Olney was the first to demonstrate the link between the neurotoxic properties of acidic amino acids and their excitatory potency [Olney 1971].

The original nomenclature of EAA receptors was based on the response to their most favored agonists, NMDA, Quisqualate (Q) and kainate (KA). Thus the glutamate excitatory receptors were delineated into three subtypes. The classification was later revised because the specificity of the agonists was not as absolute as originally thought to be. For example, Q has a high affinity for KA binding sites and will displace NMDA-like sites in micromolar concentrations. Kainate has a high affinity for Q-like sites. The new classification substituted the prototypical name of the agonists for A1 (NMDA), A2 (Q) and A3 (KA) [Foster 1986]. In this thesis the older nomenclature will be used, since the discussion will deal specifically with the NMDA subtype of glutamate receptor.

The NMDA subtype of glutamate receptor has been localized in different parts of the brain, with high concen-

trations found in the strata oriens and radiatum of the hippocampal CA1 region [Monaghan et 1983]. The CA3 zone as well as the dentate gyrus contains more moderate receptor densities. In the neocortex there is a dense band of sites corresponding to layers 1 to 3 and an additional zone in layer 5a [Cotman et al 1987].

A question often asked is how does overactivation of the NMDA receptor lead to selective neuronal death? The action of EAAs acting at NMDA receptors is characterized by two critical features, Mg^{2+} blockade which is sensitive to the membrane potential, and a coupling to a voltage-sensitive conductance. Mayer and his associates, working with cultured spinal neurons from 13 to 14 day old mouse embryos have shown that the antagonism of NMDA responses by Mg^{2+} are correlated to the membrane potential [Mayer et al 1984]. The antagonism was strong at a patch clamped holding potential of -60 mV (inward current flow), and virtually absent at +20 mV (outward current flow). The antagonistic response of the divalent cation was positively correlated to the concentration of applied NMDA. These findings strongly suggest that the Mg^{2+} ions partially enter the channels which were activated by the NMDA. This is theoretically possible because the binding of the Mg^{2+} to the channel would be influenced by the membrane electric field [Mayer et al 1984]. Further patch clamp studies of cultured embryonic mouse striatal neurons with mani-

pulation of the extracellular Mg^{2+} concentration have provided evidence that the probability of opening these channels depends on the divalent cation concentration [Nowak et al 1984]. The binding of an agonist at the NMDA receptor is insufficient to activate the conductance, because of the Mg^{2+} block. Prolonged depolarizations of the membrane which occur for example during seizure activity, or by excessive neurotransmitter release (for example during hypoglycemic isoelectricity), release the Mg^{2+} blockade and switch on the NMDA receptor channel.

The question still remains, how does activation of the NMDA receptor lead to neuronal necrosis in hypoglycemia? Experiments using cultured neurons by different groups have provided evidence which strongly suggest that damage to neurons occurs in a temporally biphasic manner. Exposure of cortical cultured neurons to 0.5 mM of L-glu in a control salt solution (CSS, containing all the required anions and cations) initially leads to an acute swelling of the neurons, followed by darkening and increased granulation of the perikaryon [Choi et al 1987]. Within 30 min these cells are necrotic. If the milieu of the CSS does not contain Na^{+}, and choline is used as a substitute, the acute swelling is not observed, but the delayed necrosis of the cells is still observed. On the other hand, if Ca^{2+} is removed from the medium, the initial swelling is observed, but the number of

cells undergoing necrosis is drastically reduced. Excluding both, Na^+ and Ca^{2+} from the CSS, acute swelling and necrosis are prevented [Choi 1987]. Thus the acute component of glutamate neurotoxicity (GNT) is Na^+ and Cl^- dependent, and the late component is Ca^{2+} dependent. In the acute component of GNT, a steady depolarization of the neuron (due to Na^+ activated channels) leads to an influx of Cl^- into the neuron [Rothman 1985]. This influx cannot be compensated by outward anion flux, because the bulk of intracellular anions are impermeant. This unstable situation causes an increased flux of cations into the cell, which increases intracellular osmolarity. This then subsequently leads to water entry [Kuffler et al 1984], thus the massive ion fluxes cause the cells to swell, and subsequent lysis occurs [Rothman 1985]. The delayed Ca^{2+} component of GNT is partially sensitive to the presence of Mg^{2+} [Garthwaite and Garthwaite 1987] in the CSS. Intracellular concentrations of Ca^{2+} of patch clamped cells have been measured spectrophotometrically with the use of the dye arsenazo III when the cells were stimulated with the application of glu. The patched membranes with potentials between -50 to -60 mV precluded the activation of voltage sensitive Ca^{2+} channels, yet there was an appreciable increase in intracellular Ca^{2+}. This evidence strongly suggests that Ca^{2+} permeability is increased, due to the opening of the NMDA channel receptor [MacDermott et al 1986].

An increased intracellular Ca^{2+} concentration when the cell is under an insult with its Ca^{2+} sequestration apparatus under less than optimal conditions can cause the activation of Ca^{2+} dependent proteases, which if not checked can cause irreversible damage to the cell [Choi 1985, Choi 1987, Choi et al 1987].

Quinolinic Acid and Neurotoxicity

Quinolinic acid (2,3-pyridine dicarboxylic acid) is a tryptophan metabolite which has been isolated in hepatic tissue and other peripheral organs. It is a precursor of nicotinamide adenine dinucleotide. In the early 1980's this compound was identified in the brain of rodents and humans [Wolfensberger et al 1983]. Moroni corroborated Wolfensberger's findings and has also shown that the brain QUIN concentration increases with age [Moroni et al 1984]. The mammalian brain is extremely sensitive to the excitatory effects of kainate and ibotenate. These excitatory and toxic amino acids are capable of producing excitotoxic lesions when injected into the brain. Intracerebral injections of QUIN in nanomolar concentrations produces axon-sparing dendritic lesions [Schwarcz et al 1983] which have the same neuropathology as the classical excitotoxins (NMDA, and the heterocyclic amino acids kainic and ibotenic). Intraventricular

injections of QUIN in mice induces seizure-like activity [Lapin 1982]. The distribution and potency of QUIN in the central nervous system (CNS) is variable. It is excitatory in some areas of the CNS such as the cortex, striatum, hippocampus and brainstem and almost completely inactive in others, such as the spinal cord and the cerebellum [Perkins and Stone 1983, Schwarcz and Köhler 1983]. This contrasts with the NMDA agonist ibotenic acid, which shows a more uniform spectrum of potency in these areas [Köhler and Schwarcz 1983].

In the rat hippocampus, QUIN acts preferentially to deplete the pyramidal neurons (particularly in the area of CA1 [Schwarcz et al 1984a], whereas the granule cells of the dentate gyrus are only destroyed at high doses [Schwarcz et al 1983]. This property is not shared by other excitotoxins including NMDA [Nadler et al 1981] which are equally toxic in both pyramidal and granule cells.

Injections of QUIN into immature 7 day old rat striatum are without effect, but the same dosage injected into mature rat striatum produce severe neuronal necrosis. The excitotoxin NMDA is equally neurotoxic in both immature and adult striatum [Foster et al 1983, Steiner et al 1984]. In profound hypoglycemia, destruction of cortico-striatal and meso-striatal input pathways into the striatum mitigate hypoglycemic brain damage [Wieloch et al 1985, Lindvall et al 1986]. The

immature rat striatum is resistant to QUIN's neurotoxicity, and this might be due to the fact that immature rat striatum do not possess complete cortico-striatal innervation. This idea is supported by the observation that removal of cortico-striatal nerve endings following unilateral decortication in the adult rat completely abolishes the neurotoxic effects of QUIN [Schwarcz et al 1984b].

Quinolinic acid-induced responses in rat hippocampal slices are antagonized by the specific NMDA antagonists 2-amino-5-phosphonopentanoate (AP5) and AP7. These observations suggest that QUIN induced depolarizations in the hippocampus are mediated by an NMDA-type excitatory amino acid receptor [Ganong and Cotman 1986]. These experimental results support Schwarcz's hypothesis as to QUIN's neurotoxicity. The apparent dependence of QUIN neurotoxicity on an excitatory amino acid-using input may indicate that the predominant action of QUIN is to increase the release of glutamate, aspartate or some related compound from the nerve terminals. The endogenous excitatory amino acid would then act on postsynaptic NMDA receptors causing neural excitation. An excess of QUIN would cause a sustained release and consequent overexcitation of the postsynaptic neuron, resulting in cell death. This scheme would explain why QUIN requires an intact excitatory amino acid input, and why QUIN excitotoxicity is blocked by NMDA antagonists.

Tryptophan loading in rats produces a marked increase in urinary excretion of QUIN [Henderson and Hirsch 1949]. A question which can now be asked is whether tryptophan loading in experimental animals can increase the brain QUIN concentration. Injections of up to 450 mg/kg of QUIN in rats produce minor seizure related activity to the EEG, but there is negligible penetration of the blood brain barrier. Direct application of [^{3}H]-QUIN on brain tissue does not indicate any substantial uptake by striatal or hippocampal synaptosomes [Foster et al 1984]. Intraperitoneal injections of the tritiated QUIN do not substantially cross the blood brain barrier. In fact only 0.3% of the acid was found to pass the blood brain barrier at a single pass [Foster et al 1984]. Histology carried out on rats which had intraperitoneal injections of QUIN, showed extremely low neuronal damage. Intrastriatal injections of the tritiated QUIN were still present 2 h after the injections, indicating that there is no probable mechanism present for the catabolism of this compound [Foster et al 1984]. The results provided by Foster and his associates indicate that QUIN is not a probable candidate as a neurotransmitter. Neurotransmitters have either an uptake or specific extracellular enzymatic processes which terminate their chemical functionality, and the adult rat brain is well protected from blood QUIN by its blood brain barrier.

Although so far no concrete evidence has been provided

regarding the role of QUIN as a neurotransmitter, Foster and Schwarcz have shown that there are both synthetic and degradadive enzymes present in the CNS responsible for QUIN synthesis and catabolism [Foster et al 1985a, Foster 1985b].

METHODS AND PROCEDURES

Hypoglycemia Model

The recovery model of insulin induced hypoglycemic coma developed by [Auer et al 1984a] was used in the following experiments. Male Wistar rats (Charles River, St. Constant, Quebec) were fasted overnight with free access to tap water. They were given 10-15 IU/kg of regular bovine insulin (Sigma, St. Louis, Mo.) intraperitoneally. The animals were anesthetized in 3.0% halothane, intubated (PE240 polyethylene tubing Clay Adams, Parsipanny, N.Y.) under ventral transillumination with a fiber optic source and then ventilated on a Starling type ventilator (Harvard Apparatus, South Natick, Mass.) with 1.0% halothane in a 2:1 N_2O:O_2 mixture.

A tail vein was cannulated (PE50 polyethylene tubing, Clay Adams) and a continuous infusion of suxamethonium chloride (2 mg/ml, Sigma) at a rate of 0.02-0.04 ml/min was administered via an infusion pump. The ventral tail artery was

cannulated (PE50, Clay Adams) and the blood pressure was recorded via a Statham transducer (Gould P50, Oxnard, Calif.) Blood pressure readings were automatically recorded to the hard disk of a computer at 6 second intervals. A bipolar interhemispheric EEG was monitored via E2 subdermal platinum scalp electrodes (Grass Instruments, Quincy, Mass.) through a universal amplifier (Gould, Cleveland, Ohio). The EEG was digitized on a computer in real time, and selected representative 5 s strips were stored to disk. A heparinized central venous catheter was inserted via the right jugular vein for the purpose of controlling the blood pressure by withdrawal and reinfusion of blood as required. Arterial blood glucose levels were monitored with an Ames glucometer (Ames, Elkhart, Ind.). Arterial blood pH, p_aCO_2 and p_aO_2 were monitored every 30 min on a blood gas analyzer (Instrumentation Laboratories 1304, Milan, Italy).

Upon completion of the operation, the animals were ventilated on a 2:1 N_2O:O_2 mixture and cerebral isoelectricity was awaited. After 30 min of isoelectricity, the animals were infused with 1.5 ml of 25% glucose solution for a period of 2 min, then the infusion rate was adjusted between 0.056-0.078 ml/min such that the blood glucose levels rapidly ascended to a plateau between 5.6 and 10.5 mM within 2 to 3 min. When the rats began to awaken 1 to 3 h later, they were weaned off the ventilator, extubated and returned to their

cages. Oxygen and intravenous glucose were given for 10 to 12 h post surgical recovery.

Neuropathology and Quantification

At one week survival, the animals were perfusion fixed. They were anesthetized in 3.0% halothane in a 2:1 N_2O:O_2 mixture, intubated and placed on a Starling type ventilator. A thoracotomy was performed and a cannula was placed in the left ventricle and secured with a suture, with its tip located in the ascending aorta. The right auricle was cut, allowing the returned perfusate from the brain to exit unhindered. After a 1 min rinse of the cerebral circulation with 0.9% saline solution, the perfusate was switched to 4% formaldehyde buffered to pH 7.35 with sodium phosphate. The animals were placed in the refrigerator and the brains removed the following day and stored in 4% formaldehyde until they were processed. The brains were then cut coronally into 2 mm slices, embedded in paraffin, subserially sectioned at 200 μm intervals, double stained with 1% acid fuchsin and 0.1% cresyl violet for the first project, (2% phloxine and 0.1% cresyl violet for the second project) and were examined under a light microscope.

The quantification of necrotic neurons was carried out blindly by direct visual counting of acidophilic neurons un-

der the microscope. Such neurons are known to be irreversibly damaged because of their cell membrane breaks, mitochondrial flocculent densities, and by their eventual disappearance from tissue sections [Auer et al 1985b]. Brain damage was graded according to the degree of neuronal necrosis. In the cerebral cortex, animals were rated according to the number of necrotic neurons per section at the coronal level of the subfornical organ, into the following grades of damage: 0-10 necrotic cells (value of 0), 11-100 necrotic cells (1), 101-1000 necrotic cells (2), and >1000 necrotic cells (3).

In the hippocampus, where the total cell counts were available from previous quantitative studies [Auer et al 1984a], the per cent neuronal necrosis in the dentate gyrus and CA1 pyramidal cells was calculated at each level of the hippocampus along its septotemporal axis.

In the caudate nucleus, quantification of necrotic neurons was carried out on two laterally located 500 µm microscopic fields per animal, representing the most damaged area of the rat striatum in hypoglycemia [Auer et al 1984a]. The data were summed for both brain hemispheres (number of necrotic neurons out of a total 130) and the per cent damage was calculated for the whole brain. For the second project, the quantification of the neuronal necrosis was slightly modified. For the cortex the damage was quantified as follows: <10 necrotic cells/section, 11-100 cells/ section, 101-1000

cells/section and >1000 cells per section. The caudate nucleus, dentate gyrus and CA1 band of the hippocampus were quantified as follows: <10% necrotic cells, 10-50% necrotic cells and >50% necrotic cells.

Statistics

For project 1, the derived per cent data for the hippocampus and striatum were transformed using the standard arcsine transformation [Zar 1974] and a One-Way Analysis of Variance (ANOVA) with Dunnett's 't' test was carried out between control and MK-801 means [Neter et al 1985]. The statistical evaluation used for the cortex was the non-parametric Kruskal-Wallis test with tied ranks, since the data obtained from the cortex were ranked according to graded damage. A non-parametric multiple comparison test was used to test for significance between the groups.

In the second project values presented are either mean ± S.E.M. or expressed as a percent of pooled control and sham-operated rats. Results were analysed by One-Way ANOVA and Dunnett's 't'test.

For the third project data were analysed with One-Way ANOVA and Dunnett's 't'test. The data for table three were analysed with the non-parametric Kruskal-Wallis test with

tied ranks. Since there was no significance at this stage of the analysis no further tests were required.

Project 1: The Effect of MK-801 on Structural Brain Damage

MK-801 is a potent anticonvulsant, and also a non-competitive antagonist at NMDA receptors, which readily crosses the blood brain barrier after parenteral administration. Recent experimental evidence has provided support for the excitotoxic theory of hypoglycemic brain damage [Auer et al 1985c, Wieloch 1985, Sandberg et al 1986]. The NMDA subtype of glutamate receptor is of particular interest in hypoglycemic brain damage. Competitive and non-competitive antagonists of these receptors protect neurons in several brain regions against the adverse effects of excitotoxins. Simultaneous injections of the endogenous excitotoxin QUIN and an NMDA non-competitive antagonist, 2-amino-7-phosphonoheptanoic acid (AP7), mitigates striatal neuronal necrosis [Schwarcz et al 1983, Schwarcz et al 1984b]. The excitatory amino acid receptors involved in mediating hypoglycemic neuronal necrosis are of the NMDA subtype [Wieloch 1985].

MK-801 is the most potent non-competitive NMDA antagonist reported to date [Wong et al 1986]. It blocks the NMDA channel in a use-dependence manner [Davies et al 1988] which

inhibits inward cationic fluxes through the receptor associated channel.

MK-801 presumably mitigates neuronal damage by preventing the delayed Ca^{2+} component of the NMDA mediated neurotoxicity. Several other research groups have postulated that MK-801 attenuates neuronal necrosis via a hypothermic mechanism [Busto et al 1987, Davies et al 1988]. Given the properties of MK-801, the first project was designed to illustrate the efficacy of this non-competitive antagonist in the mitigation of hypoglycemic brain damage.

Four groups (n=6) of Male Wistar rats, weighing 222-466 g were used. Three treatment groups were given MK-801 in intravenous doses of 1.5, 2.8 and 5.0 mg/kg, injected iv during the high amplitude slowing of the EEG to delta range, while control rats were given (0.9%) saline. Cerebral isoelectricity was maintained for 30 min. Doses greater than 5.0 mg/kg usually resulted in a high mortality rate. The two lower doses were chosen so as to describe the dose-response relationship. The animals were recovered and allowed to survive 1 week prior to sacrifice.

Results

A significant clinical difference was seen between the untreated rats and the rats treated with MK-801. The control

animals were the easiest to wean off the respirator, usually within 1 to 2 h after reconstitution to normoglycemia. Animals treated with MK-801 required longer ventilatory assistance. Mean arterial blood pressure of the MK-801 treated animals was consistently higher than control animals during the recovery period (Table 1; $F=10.37$, $p<0.001$). All three doses of MK-801 produced catalepsy, defined as a wakeful state of immobility with the eyes open, from which the rats could not be roused or stimulated. The rats lay on their side with the eyes open, unable to right themselves. This cataleptic state gradually subsided over the course of the first 2 to 3 days of survival. The 5.0 mg/kg animals required the longest time to recuperate.

Neuropathologically, MK-801 decreased hypoglycemic brain damage in the hippocampus, striatum, and cortex. The mitigation of damage in the CA1 sector was dose dependent at the higher doses (Figure 1) ($F=7.854$, $p<0.0025$). The mean per cent necrosis for each group was as follows; control, 32.5 ± 6.3 (S.E.M.); 1.5 mg/kg MK-801, 23.5 ± 2.9; 2.8 mg/kg MK-801, 10.8 ± 2.4 and 5.0 mg/kg MK-801, 8.5 ± 1.3. Comparison of the control mean to the three MK-801 dose means showed a statistical difference at the 2.8 and 5.0 mg/kg MK-801 doses $p<0.05$ and $p<0.01$ respectively; Dunnett's multiple comparison test. Histologically, the reduction in neuronal necrosis in the CA1 pyramidal cell band was apparent on visual examina-

tion (Figures 2, 3).

In the dentate gyrus, all doses of MK-801 mitigated neuronal necrosis (Figure 4), ($F=16.632$, $p<0.001$). The mean per cent necrosis for each group was as follow; control, 8.9 ± 1.0; 1.5 mg/kg MK-801, 5.4 ± 0.5, 2.8 mg/kg MK-801, 6.6 ± 0.8 and 5.0 mg/kg MK-801, 2.4 ± 0.3. The dentate granule cell band, which is very susceptible to hypoglycemic brain damage, was in frequent sections completely protected with the 5.0 mg/kg MK-801 dose (Figures 5, 6). Comparison of control mean to the three dose means has shown a statistical difference between the control and drug treated groups; 1.5 mg/kg, $p<0.01$; 2.8 mg/kg $p<0.05$; 5.0 mg/kg, $p<0.01$.

In the lateral caudate nucleus, neuronal necrosis was reduced from 76.2% of the neuronal population to 5.6% in animals treated with 5.0 mg/kg of MK-801 ($F=7.14$, $p<0.005$) (Figure 7). The mean per cent neuronal necrosis for the different groups is as follow; control, 76.2 ± 7.1; 1.5 mg/kg MK-801, 26.8 ± 7.6; 2.8 mg/kg MK-801, 35.1 ± 10.9 and 5.0 mg/kg MK-801 5.6 ± 4.3. Complete histologic protection against neuronal necrosis was commonly seen with the highest dose (5.0 mg/kg) of MK-801 (Figures 8, 9). Multiple comparisons between control and MK-801 means revealed a statistical difference between control and MK-801 groups; 1.5 mg/kg, $p<0.05$; 2.8 mg/kg, $p<0.05$; and for the 5.0 mg/kg, MK-801 group $p<0.01$.

The cortex showed significant protection between control and 2.8 mg/kg of MK-801 (Figure 10). The data for each series of rats were pooled and the non-parametric Kruskal-Wallis ($p<0.05$) with tied ranks was used to test for significance. A non-parametric multiple comparison test revealed a statistical difference between control the 2.8 mg/kg MK-801 group; $p<0.01$.

Groups showed a slight decrease in core body temperature at the onset of EEG isoelectricity (Figure 11), but there was no correlation between dosage of MK-801 and the magnitude of the temperature decrease ($F=5.183$, $p<0.02$). The only group in which the body temperature was significantly lower than control was the 1.5 mg/kg group (Dunnett's `t' test $p<0.01$), the group showing the least robust neuroprotection histologically.

Table 1: Physiologic measures during insulin induced hypoglycemia and in the recovery period following glucose administration. One-Way ANOVA, Dunnett's 't' test between control and experimental group means was significant where indicated; * $p<0.05$. MABP, mean arterial blood pressure; Iso, period of isoelectricity; post, Post isoelectric period.

Table 1:

Physiologic Measures during Insulin Induced Hypoglycemia and in the Recovery Period Following Glucose Administration

	Control	1.5 mg/kg	2.8 mg/kg	5.0 mg/kg
MABP (iso)	117.3 ± 5.8	118.7 ± 7.0	106.3 ± 10.5	117.3 ± 6.5
MABP(post)	108.9 ± 2.5	*143.3 ± 2.6	*140.2 ± 4.3	*135.9 ± 3.6
Body Temperature (°C)	37.5 ± 0.1	37.3 ± 0.1	37.2 ± 0.3	37.5 ± 0.0
Blood Glucose (iso)	0.9 ± 0.0	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.0
Blood Glucose (post)	7.6 ± 1.0	5.5 ± 0.6	7.9 ± 1.1	9.2 ± 1.1
pH(iso)	7.38 ± 0.02	7.44 ± 0.03	7.46 ± 0.03	7.42 ± 0.02
pH(post)	7.37 ± 0.02	7.35 ± 0.02	7.36 ± 0.03	7.35 ± 0.01
pCO_2(iso)	31.4 ± 2.2	25.6 ± 1.2	26.9 ± 1.8	30.3 ± 1.5
pCO_2(post)	35.8 ± 1.7	34.9 ± 1.7	30.4 ± 1.2	35.0 ± 1.2
pO_2(iso)	101.0 ± 2.3	109.0 ± 9.1	114.0 ± 10.5	*137.0 ± 5.7
pO_2(post)	108.0 ± 3.2	109.0 ± 4.7	119.0 ± 5.8	*131.0 ± 5.8

Figure 1: Percent neuronal necrosis in the CA1 sector of the hippocampus following injection of MK-801 (n=6). Bars denote mean ± S.E.M. Percentages were transformed according to the standard arcsine transformation [Zar 1974]. One-Way ANOVA with Dunnett's `t` test between control and experimental group means was significant; * $p<0.05$, ** $p<0.01$.

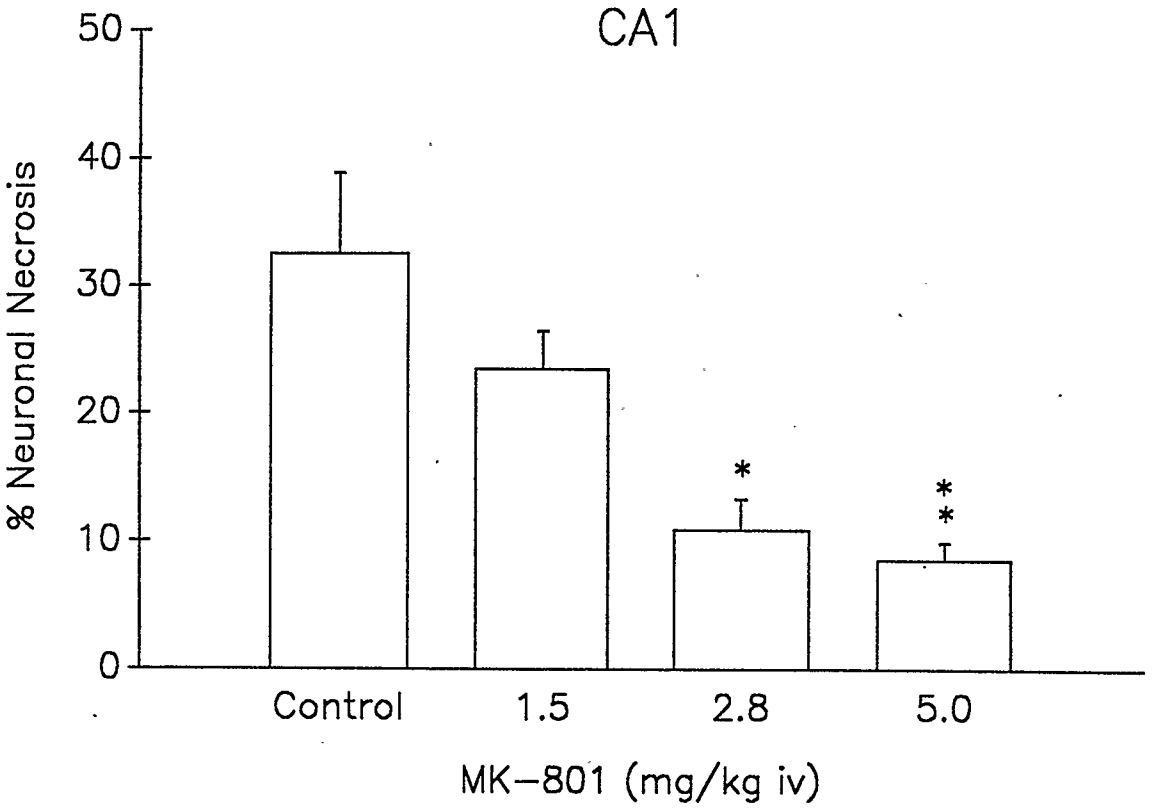

CA1
% Neuronal Necrosis
50
40
30
20
10
0
*
**
Control
1.5
2.8
5.0
MK-801 (mg/kg iv)

Figure 2: Hippocampal neuronal necrosis in rats subjected to 30 min of cerebral isoelectricity due to profound hypoglycemia. Untreated rats show extensive neuronal necrosis along the CA1 pyramidal cell band. 1% Acid fuchsin, 0.1% cresyl violet. Bar = 200μm.

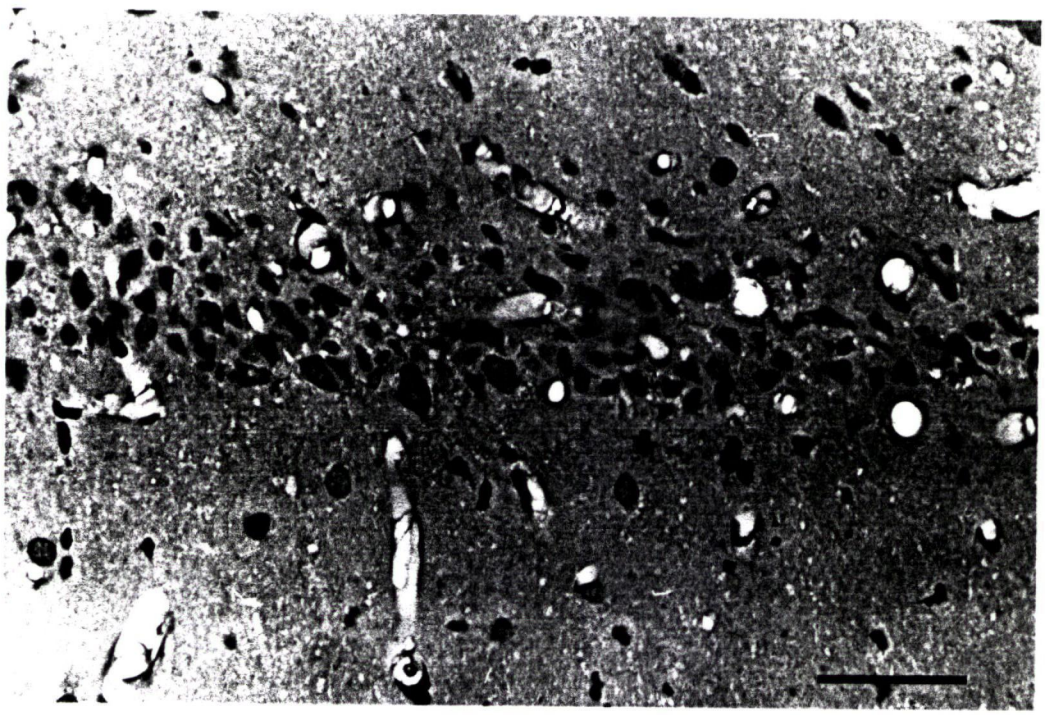


Figure 3: Hippocampal neuronal preservation in rats subjected to 30 min of isoelectricity due to profound hypoglycemia, but treated with 5.0 mg/kg iv MK-801. 1% Acid fuchsin, 0.1% cresyl violet. Bar = 200μm.

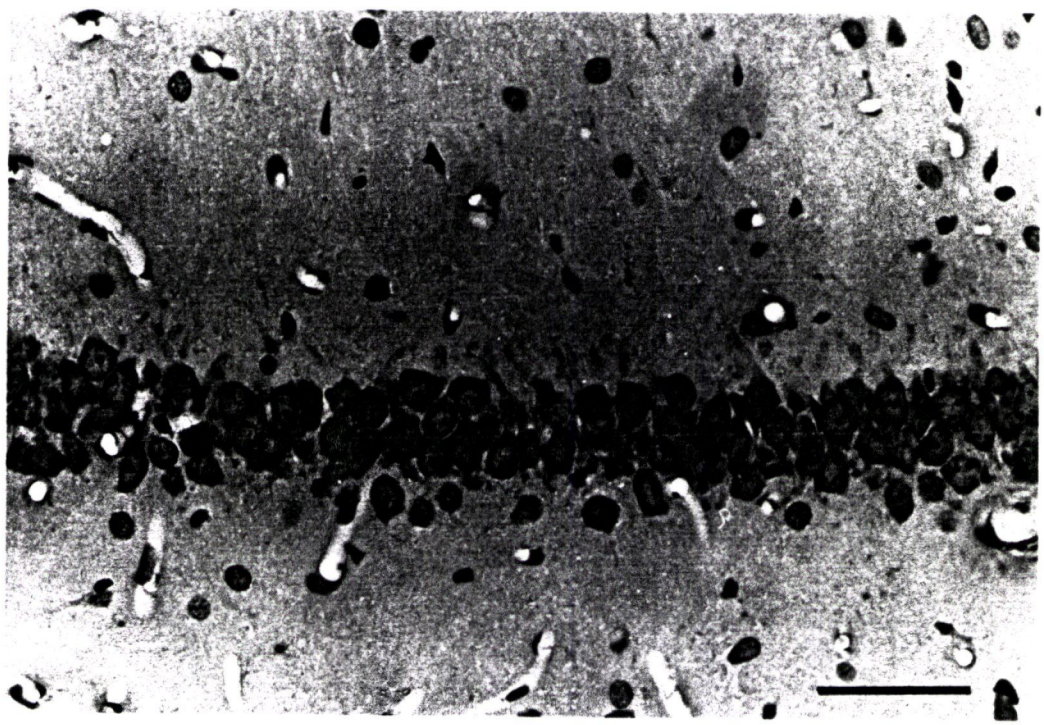


Figure 4: Percent neuronal necrosis in the dentate gyrus of the hippocampus following injections of MK-801 (n=6). Bars denote mean ± S.E.M. Percentages were transformed according to the standard arcsine transformation [Zar 1974]. One-Way ANOVA with Dunnett's multiple comparison test between control and experimental means was significant; * $p<0.05$, ** $p<0.01$.

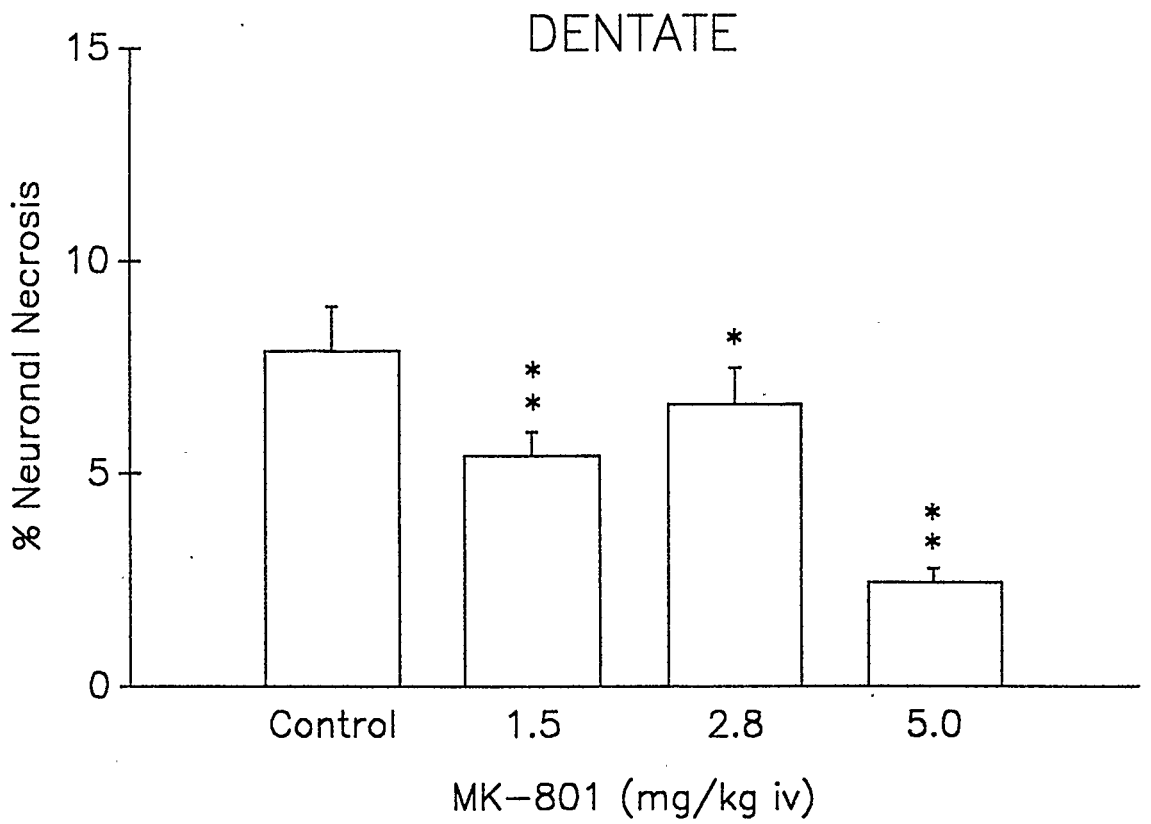

DENTATE
% Neuronal Necrosis
15
10
5
0
**
*
**
Control
1.5
2.8
5.0
MK-801 (mg/kg iv)

Figure 5: The dentate gyrus in an untreated rat. The crest of the dentate gyrus is visible on the right of the photomicrograph, with the internal and external blades of the dentate in the superior and inferior portion of the photomicrograph. Necrotic neurons have pyknotic, shrunken nuclei, seen in the crest and outer blade; one week after 30 min of cerebral EEG isoelectricity due to profound hypoglycemia. 1% Acid fuchsin 0.1% cresyl violet. Bar = 125μm.

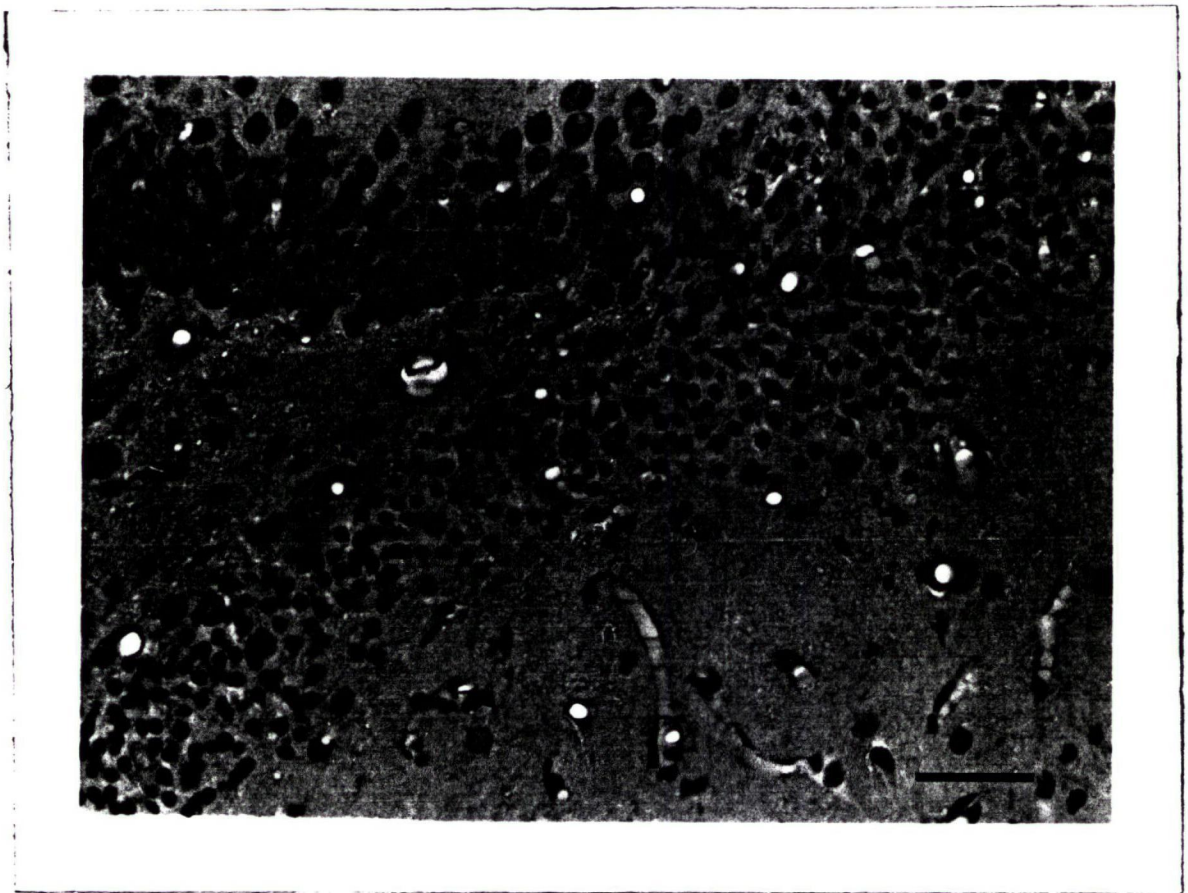


Figure 6: The dentate gyrus of a rat treated with 5.0 mg/kg iv of MK-801 at a comparable septotemporal level of the hippocampus. There is essentially no neuronal necrosis in the crest or the external blade of the gyrus. 1% Acid fuchsin, 0.1% cresyl violet. Bar = 125μm.

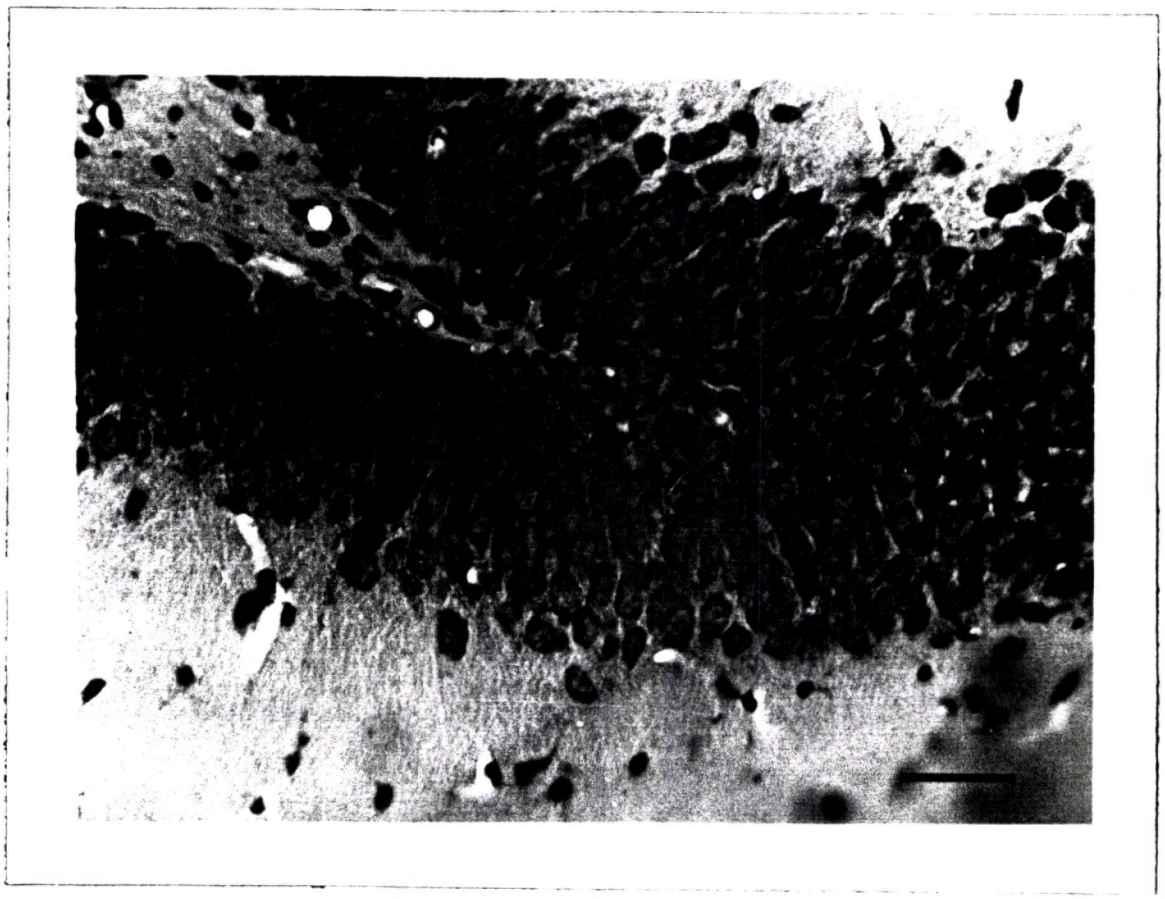


Figure 7: Percent neuronal necrosis in the caudate nucleus following injections of MK-801 (n=6). Bars denote ± S.E.M. Percents were transformed by the standard arcsine transformation [Zar 1974]. One-Way ANOVA with Dunnett's multiple comparison test between control and experimental group means was significant; * $p<0.05$, ** $p<0.01$.

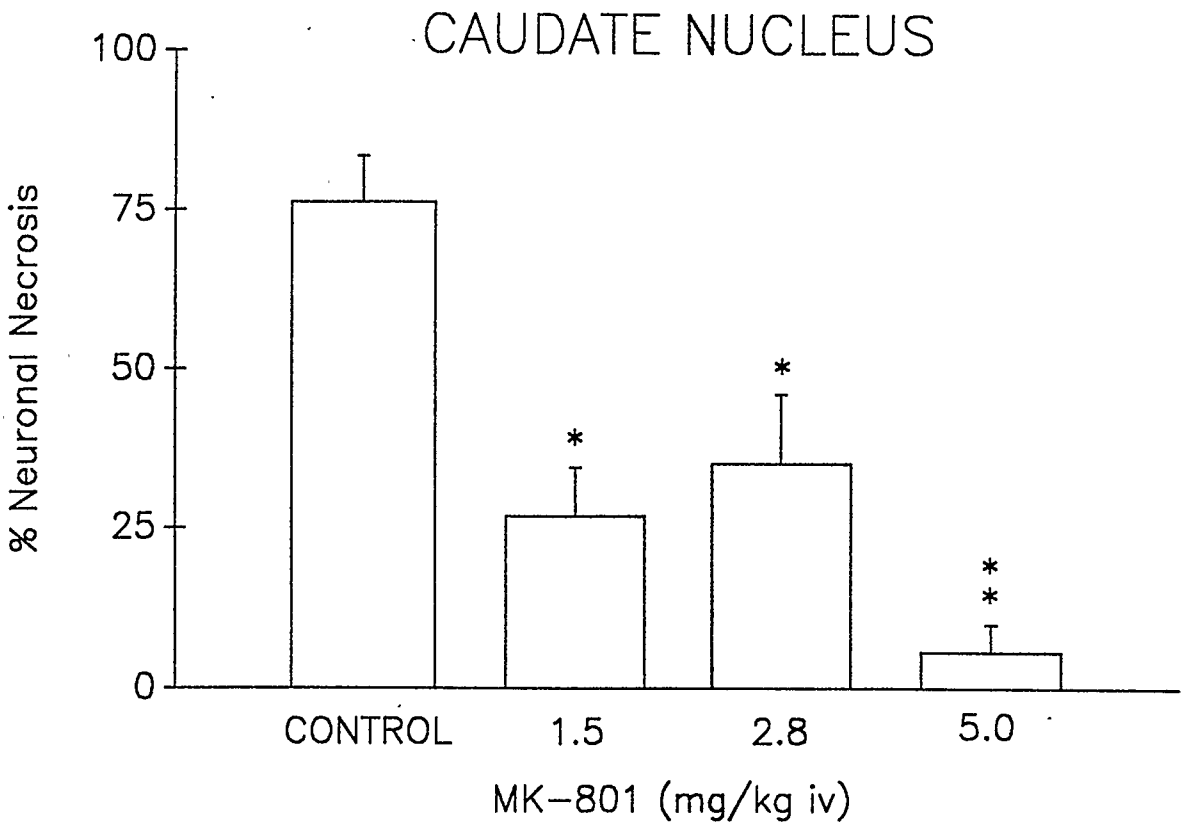

CAUDATE NUCLEUS
% Neuronal Necrosis
100
75
50
25
0
*
*
**
CONTROL
1.5
2.8
5.0
MK-801 (mg/kg iv)

Figure 8: The caudate nucleus of a rat which underwent 30 min of EEG isoelectricity due to profound hypoglycemia. After 1 week survival, intact neurons are few and sparse. 1% Acid fuchsin, 0.1% cresyl violet. Bar = 120μm.

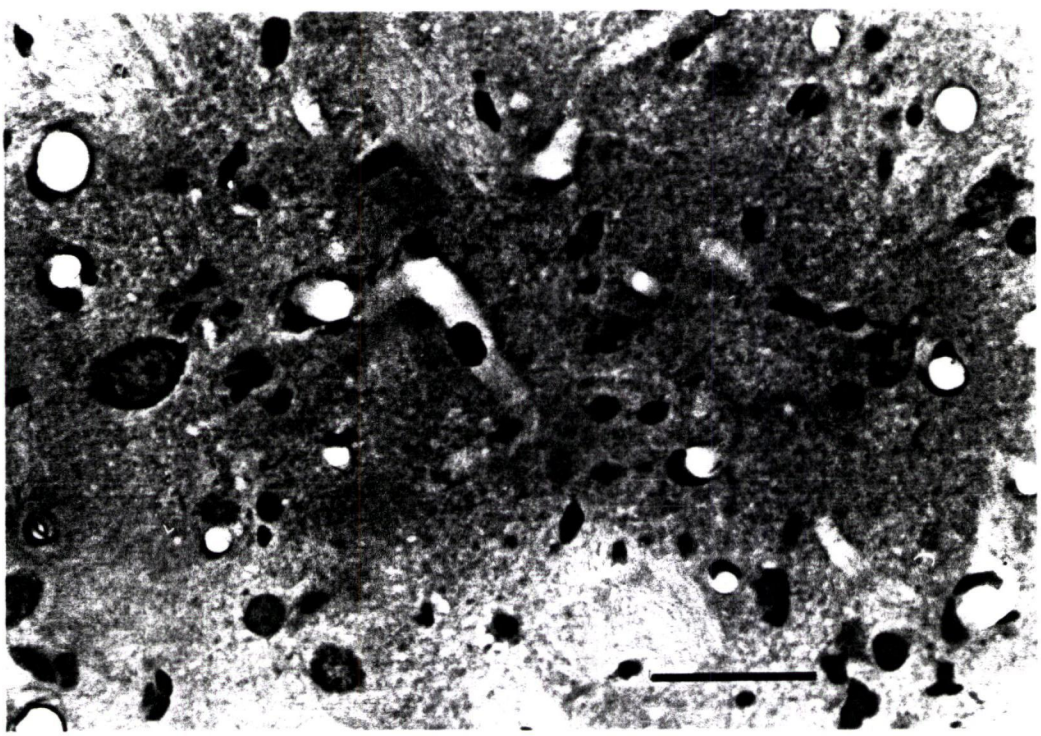


Figure 9: The caudate nucleus showed a marked preservation of neurons by MK-801 5.0 mg/kg iv. The rats were subjected to 30 min of cerebral isoelectricity due to profound hypoglycemia. Histology was done 1 week post surgical survival. 1% Acid fuchsin, 0.1% cresyl violet. Bar = 120μm.

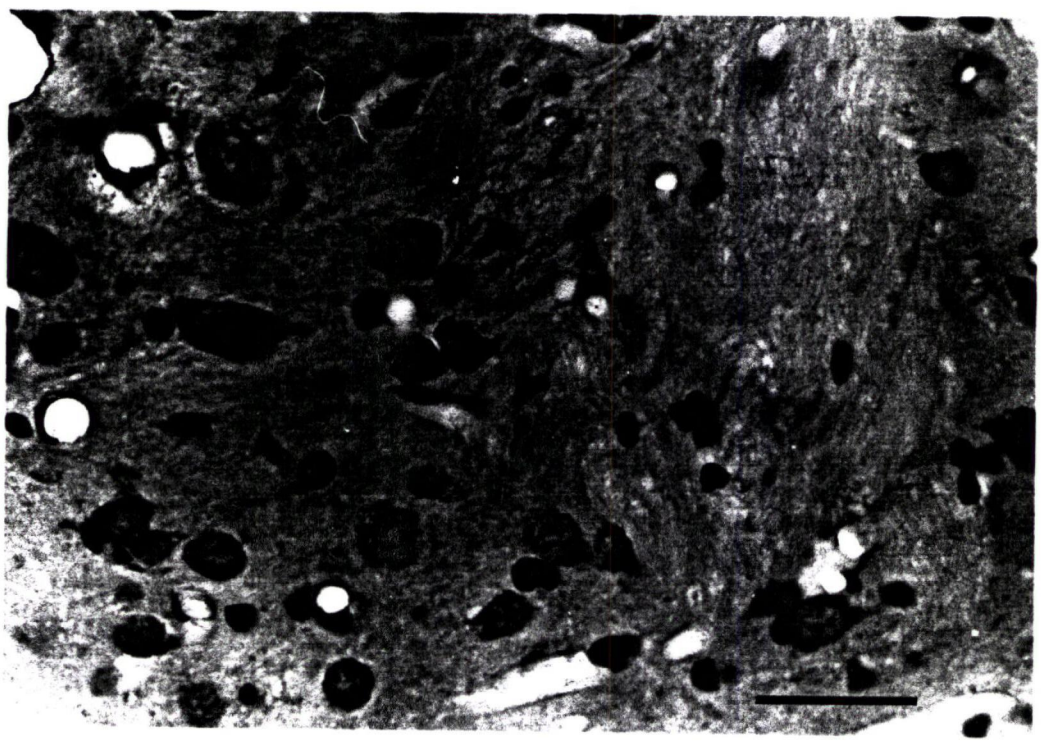


Figure 10: The number of hemispheres exhibiting the indicated numbers of necrotic neurons in the control and three MK-801 treated groups (n=6). The pooled data from individual groups showed a statistical difference from control; Kruskal-Wallis with tied ranks, $p<0.05$. A non-parametric multiple comparison test between group means revealed a statistical significance between the control and 2.8 mg/kg MK-801 group, $p<0.01$.

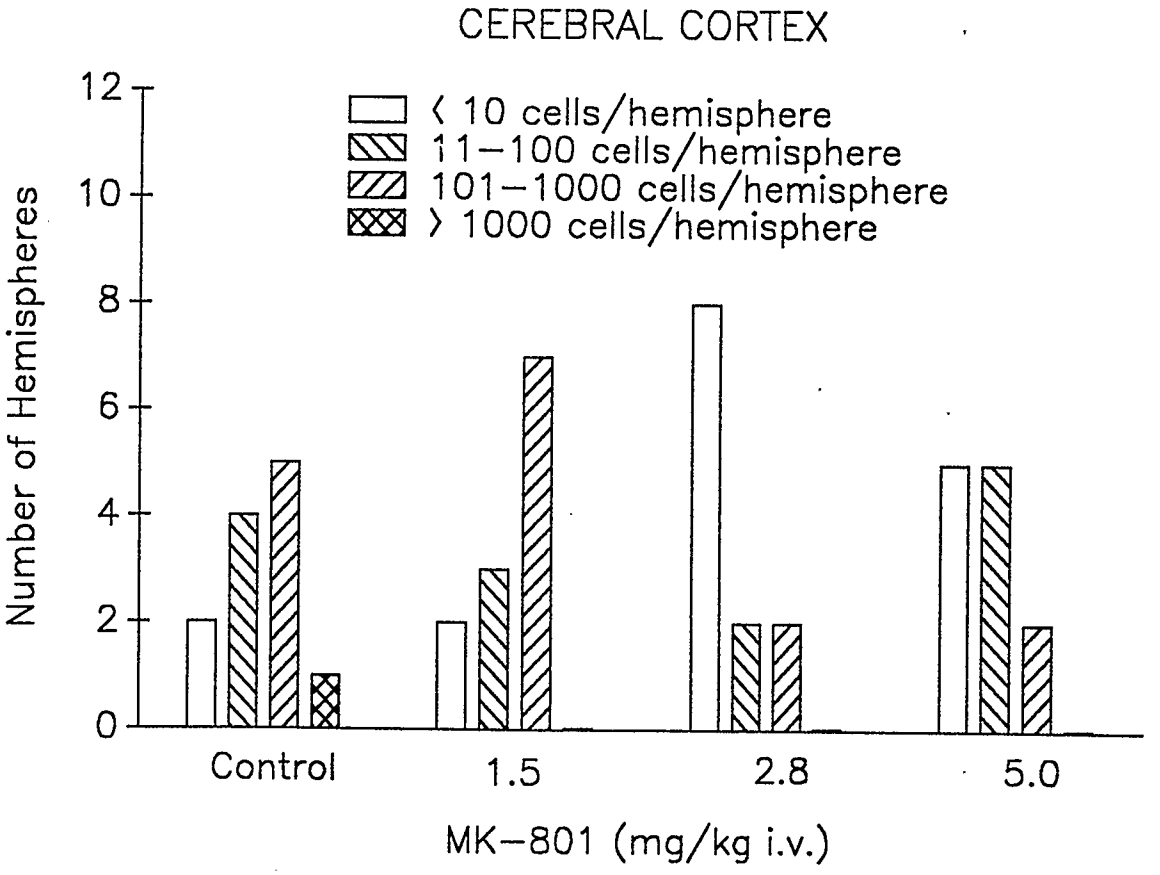
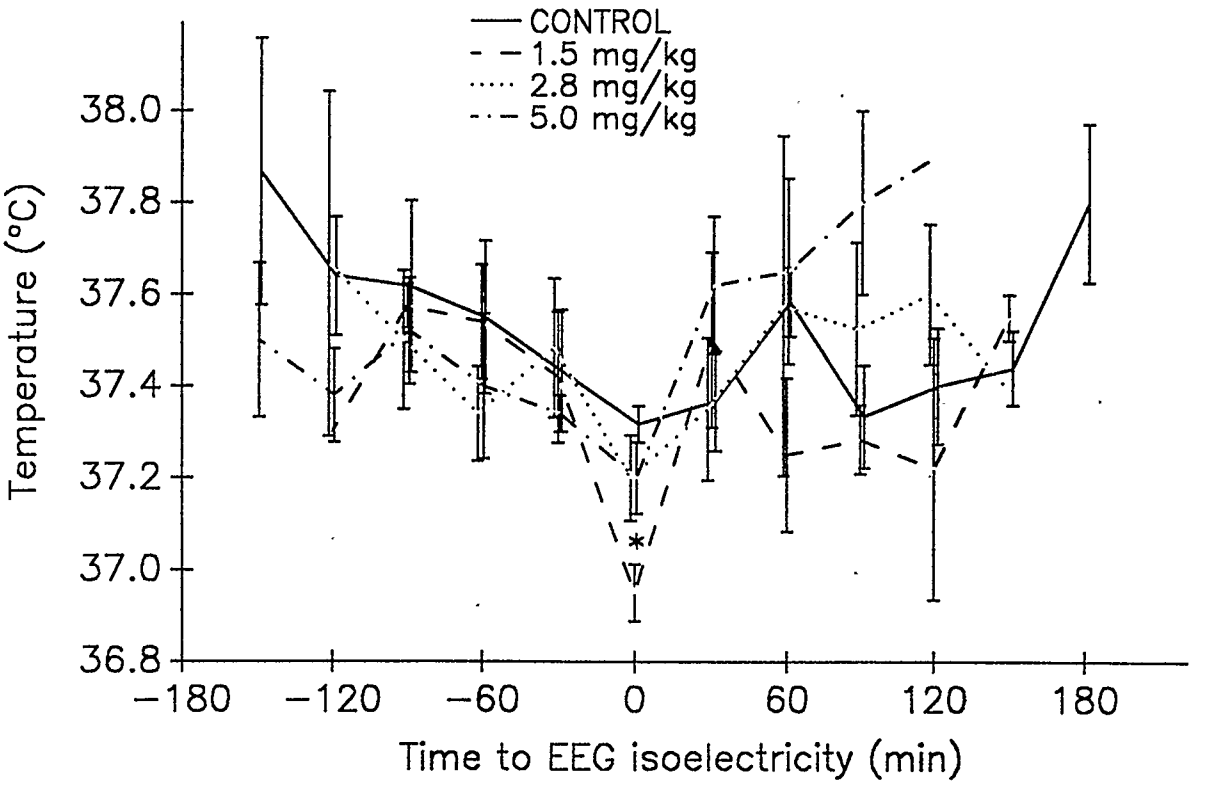

CEREBRAL CORTEX
〈 10 cells/hemisphere
11–100 cells/hemisphere
101–1000 cells/hemisphere
〉 1000 cells/hemisphere
Number of Hemispheres
0
2
4
6
8
10
12
Control
1.5
2.8
5.0
MK–801 (mg/kg i.v.)

Figure 11: Core body temperature as a function of time in relation to the onset of cerebral EEG silence (time 0 on x-axis). Recordings were taken at 30 min intervals from 3 h prior to isoelectricity to a maximum of 3 h recovery. Only the temperature fall in the 1.5 mg/kg group was significantly different from control, and only at the onset of isoelectricity; One-Way ANOVA with Dunnett's `t' test; * $p<0.01$).


CONTROL
1.5 mg/kg
2.8 mg/kg
5.0 mg/kg
Temperature (°C)
38.0
37.8
37.6
37.4
37.2
37.0
36.8
−180
−120
−60
0
60
120
180
Time to EEG isoelectricity (min)

Discussion

The present findings indicate that neuronal necrosis in the brain may be reduced by intravenous administration of the centrally penetrating NMDA antagonist MK-801 at the stage of profound hypoglycemia accompanied by delta wave slowing of the EEG, even if coma (isoelectric EEG, electro-cerebral silence) ensues.

There is a marked increase in brain aspartate levels during hypoglycemia [Agardh et al 1981b]. Aspartate is an excitatory amino acid [Fonnum et al 1983] and gains access to the extracellular space of the brain in hypoglycemia [Sandberg et al 1986] where neuronal receptors are located. These findings make aspartic acid an attractive candidate in the pathogenesis of hypoglycemic neuronal necrosis.

MK-801 is one of the most potent excitatory amino acid receptor antagonists known, and binds with extreme affinity to isolated brain fractions [Wong et al 1986]. The compound was first described in 1982 to have anticonvulsant, sympathomimetic and anxiolytic activity [Cleinschmidt et al 1982a; Cleinschmidt et al 1982b; Cleinschmidt et al 1982c].

MK-801 has been thought to act via a hypothermic mechanism. Hypothermia is known to be protective against hypoxic-ischemic neuronal necrosis [Busto et al 1987]. Some temperature-dependence of blockade by MK-801 has been shown

[Davies et al 1988]. To address this question, body temperature was monitored continuously in this study, and was found to fall only slightly at the onset of isoelectricity. This fall in body temperature was only significant in the 1.5 mg/kg group, where the smallest neuroprotective effect was seen. Furthermore, since the brain is well perfused during hypoglycemia [Abdul-Rahman et al 1980], the brain temperature would be expected to be near body core temperature. Thus, since the body temperature did not fall significantly in the groups most protected by MK-801, the findings of the present study argue strongly against a hypothermic mechanism of action to explain the neuroprotection seen with MK-801.

MK-801 has also been found to be experimentally effective in reducing ischemic neuronal necrosis. The various animal models in which MK-801 has been shown to reduce structural brain damage included neonatal hypoxia [Macdonald et al 1987], and both focal [Ozyurt et al 1986] and global [Gill et al 1987] ischemia. A delayed neuronal necrosis is not observed in the recovery period after hypoglycemia, as is seen in ischemia [Kirino et al 1982].

Neuronal necrosis occurs within a few h of the insult [Auer et al 1985a, Auer et al 1985b]. However, future studies are necessary to determine whether parenteral MK-801 is effective when administered at later stages of profound hypoglycemia, ie. when cerebral isoelectricity and coma have

already occurred.

Project 2: Quinolinic Acid, Tryptophan Metabolites, Indoleamine and Catecholamine Concentrations in Hypoglycemia and Recovery

Quinolinic acid is an extremely potent excitotoxin in nanomolar quantities when injected in the striatum [Schwarcz et al 1983]. Its precursor substrate is the essential amino acid L-TRP. The capillary endothelia that comprise the blood brain barrier contain two specific transport systems for neurotransmitter precursors, one for choline, and another for the transport of large neutral amino acids such as tryptophan, tyrosine, threonine, and branched chain amino acids such as leucine, isoleucine, and phenylalanine [Wurtman et al 1981]. The carrier system is not energy dependent nor can it support a concentration gradient. Due to the fact that the substrates are competitive for the same carrier loci, a variation in plasma amino acid concentrations would generally lead to differential brain amino acid concentrations passing through the blood brain barrier.

In this project the hypothesis was tested whether insulin-induced hypoglycemia would cause an increase in brain QUIN and other tryptophan metabolites. Following 40 min of cerebral isoelectricity there is an increase in the cerebral tryptophan concentration in the cerebral cortex, striatum and

limbic regions [Agardh et al 1979]. Since QUIN is a tryptophan metabolite, an increased cerebral tryptophan concentration could possibly produce an increase in the cerebral QUIN concentration. To investigate whether QUIN is increased by hypoglycemia, this study was designed to study QUIN and related compounds in brain tissue during both 40 min of profound hypoglycemia and hypoglycemia followed by 1-2 h recovery with normoglycemia. Brain homogenate measures do not indicate whether QUIN is localized predominantly in the intracellular or extracellular compartment, or is homogeneously distributed. Tissue distribution is an important consideration because the NMDA receptors, which mediate QUIN neurotoxicity, are most likely localized on the surface of neurons accessible from the extracellular space. It is possible that increases in QUIN in brain tissue during and after hypoglycemia are of sufficient magnitude to contribute to the degree of NMDA receptor activation and therefore neuronal damage. To further investigate this, a microdialysis probe was placed in the hippocampus of rat brains, and samples were collected, along with blood plasma at different time intervals and analyzed for QUIN.

The recovery model for insulin-induced hypoglycemia was used in this experiment. The mass spectroscopic and chromatographic analyses were carried out at the National Institute of Mental Health, Bethesda Md. by Dr. M. P. Heyes

[Heyes and Markey 1988a]. Four groups of six rats were studied. Control group: sacrificed on removal from their home cage. Sham operated group; hypoglycemia group: exposed to 40 min of EEG isoelectricity; recovery group: 40 min of EEG isoelectricity followed by 1 h of normoglycemic recovery. In studies of plasma, additional rats were sacrificed 1 h and 2 h after injection of insulin but prior to EEG isoelectricity and killed 1 and 2 h after beginning normoglycemic recovery from 40 min of isoelectricity. At least 5 rats per time point were studied.

The dialysis fibers were manufactured by mounting cellulose acetate hollow tubing (Molecular weight cut-off 6,000; Spectrum Medical Industries, L.A. Calif.) onto 30 gauge stainless steel wire. The ends of the fiber were then fixed to a hollow twin barrel stainless steel cannula with epoxy cement. The length of the dialysis tubing available for exchange with the interstitial fluid was 5 mm.

On the day prior to the hypoglycemic insult, animals were anesthetized by an intraperitoneal injection of 5 mg/kg pentobarbital (M.T.C. Pharmaceuticals, Mississauga, Ont.). A superior parasagittal craniotomy (2.0 mm lateral; 3.8 mm posterior to bregma) was performed with a dental burr. The cannula was lowered 3.5 mm below the skull surface into the hippocampus and cemented into place with self-curing plastic (Formatray, Kerr, Romulus, MI). The animals were allowed to

awaken and were fasted overnight prior to the induction of hypoglycemia.

On the day of hypoglycemia, the two barrels of the dialysis cannula were connected to vinyl tubing and perfusion of the probes with artificial CSF (NaCl, 0.152 M; KCl, 0.00297 M; $CaCl_2$, 0.00133 M and $MgCl_2.6H_2O$ 0.00167 M (in demineralized H_2O) was begun at 1 µl/min. Dialysates were collected every hour during control and recovery periods and at 40 min during isoelectricity by clamping the ends of the tubing into 10 cm lengths.

Cerebral isoelectricity began 60 to 170 min after the injection of insulin. After 40 min of isoelectricity, normoglycemic recovery was induced with an infusion of 1.5 ml of 25% glucose over a 2 min period followed by continuous infusion of glucose at 0.056-0.078 ml/min for 1 or 2 h. Rats were decapitated under anesthesia, their brains quickly removed and cooled on a glass plate. The frontal (50-90 mg), parietal (60-100 mg), occipital (50-90 mg) regions of cerebral cortex, striatum (25-40 mg), thalamus (30-50 mg), hippocampus (20-30 mg) and cerebellum (180-240 mg) were rapidly dissected and collected into pre-weighed 1.5 ml polypropylene tubes. The tubes were frozen in liquid nitrogen, weighed and stored at -70°C until analysis.

Neurochemical Analyses

All brain samples were sonicated in 1 ml of 1 mol/l perchloric acid ($HClO_4$; PCA) containing 6 pmol/ml [^{18}O]-QUIN as internal standard for quantification of QUIN. For the standard curve, 1 ml of the [^{18}O]-QUIN in PCA solution was added to QUIN acid standards (0.6 to 60 pmol) dissolved in water. PCA extracts were centrifuged at 12,000 xg for 20 min at 4°C. A 50 µl aliquot of the supernatant from the brain was collected into 250 µl polypropylene tubes and used for analysis of indoleamines and catecholamines by high performance liquid chromatography (HPLC). In 2 rats, 3-hydroxyanthranilic acid (100 nmol/l) was perfused through the microdialysis tube for 1 h beginning after a 2 h control period and followed by 3 h of collection.

The remaining PCA extract was used for the quantification of QUIN. Plasma (10 µl) and microdialysate fluid was mixed with 0.5 ml of 1 mol/l hydrochloric acid (HCl) or deionized water containing 6 pmol [^{18}O]-QUIN, centrifuged at 12,000 xg for 20 min at 4°C. Microdialysates were flushed from the tubing using deionized water and 0.6 pmol of [^{18}O]-QUIN added. All extracts and dialysates were freeze dried overnight for the quantification of QUIN.

Results

No significant differences were found between the sham, hypoglycemic or recovery groups with respect to body temperature, blood gases, blood pH or mean arterial blood pressure. These physiologic parameters were within the following ranges: Body temperature, 36.7-37.8°C; arterial p_aO_2, 88-145 mm Hg; arterial p_aCO_2, 31-46 mm Hg; arterial pH, 7.36-7.46 and mean arterial blood pressure 100-120 mm Hg. The two hypoglycemia groups showed a transient rise in mean arterial pressure to approximately 110-150 mm Hg during the isoelectricity period.

Plasma QUIN Concentrations

Plasma QUIN concentrations were 273.2 ± 32.1 pmol/ml in control rats. Plasma QUIN increased significantly by 2 h after injection of insulin by 3.57-fold and increased further during hypoglycemic isoelectricity to 6.47-fold higher than control. Plasma QUIN concentrations began to return to control values during recovery period, but were still increased 3.96-fold at 2 h (Figure 12).

Regional Brain QUIN, L-TRP, 5-HT and 5-HIAA Concentrations

There were no significant differences in regional brain concentrations of QUIN, L-TRP, 5-HT or 5-HIAA between control and sham rats. Likewise in striatum, the concentrations of DA, HVA and DOPAC were not different between control and sham operated rats. Therefore, the data from control and sham rats were pooled for statistical comparisons to hypoglycemia and recovery groups. The values for regional brain QUIN are presented in Figure 13. In all regions, QUIN concentrations increased 2 to 3-fold after hypoglycemia and increased 2 to 3-fold further during recovery. The regional brain concentrations of L-TRP was not changed after hypoglycemia but was increased significantly 1.5 to 2-fold in all regions after 1 h of recovery (Figure 14). Regional brain 5-HT concentrations decreased significantly after hypoglycemia by 40 to 60% (Figure 15). During the recovery period, 5-HT concentrations increased compared to hypoglycemia values, but in cortical regions, were still significantly lower than control and sham. Regional brain 5-HIAA concentrations increased significantly after hypoglycemia by 76 to 161% and were still increased after 1 h of recovery in all regions examined (Figure 16).

In striatum (Figure 17), DA concentrations decreased by 74% after hypoglycemia while at the same time, HVA and DOPAC

concentrations increased by 252% and 281% respectively. After recovery, DA concentrations had returned to control values, whereas both HVA and DOPAC concentrations remained elevated at 200% and 270% respectively above control and sham values.

In Vivo Microdialysate QUIN Concentrations

The concentrations of QUIN in microdialysate of control samples collected from hippocampus was 20 ± 4 nmol/l. No significant change in microdialysate QUIN concentrations were observed during either cerebral isoelectricity or the recovery period (Figure 18) indicating that extracellular fluid QUIN concentrations were maintained at control values throughout the study. Addition of 3-hydroxyanthranilic acid, a precursor of QUIN, to the microdialysis perfusion medium resulted in increased microdialysate QUIN concentrations of 18-fold and 13-fold in the two rats studied.

Figure 12: Plasma QUIN concentrations in control (open bar), and increased QUIN concentrations 1 h and 2 h (stippled bars) after injection of insulin, 40 min of hypoglycemic isoelectricity (closed bar) and 1 h and 2 h of normoglycemic recovery (hatched bars). Values presented are mean ± S.E.M. of at least 5 rats. One-Way ANOVA with Dunnett's 't' test; * $p<0.01$.

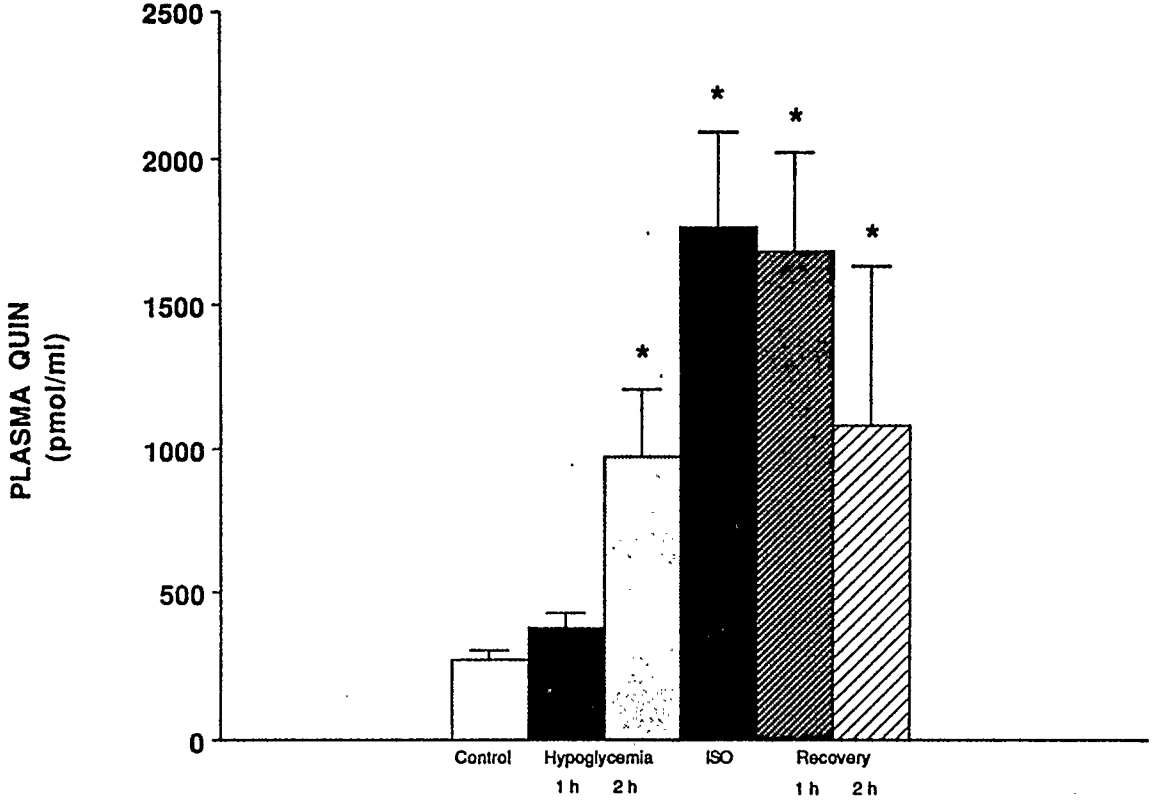

PLASMA QUIN
(pmol/ml)
2500
2000
1500
1000
500
0
*
*
*
*
Control
Hypoglycemia
1 h
2 h
ISO
Recovery
1 h
2 h

Figure 13: Regional brain QUIN concentrations in control un-operated rats (open bars), sham-operated rats (stippled bars), rats killed immediately after 40 min of insulin-induced hypoglycemic isoelectricity (hatched bars) and rats exposed to 60 min of normoglycemic recovery (filled bars). The data show apparent increases in regional brain QUIN during isoelectricity and recovery. Values presented are mean ± S.E.M. One-Way ANOVA with Dunnett's `t' test; * $p<0.01$.

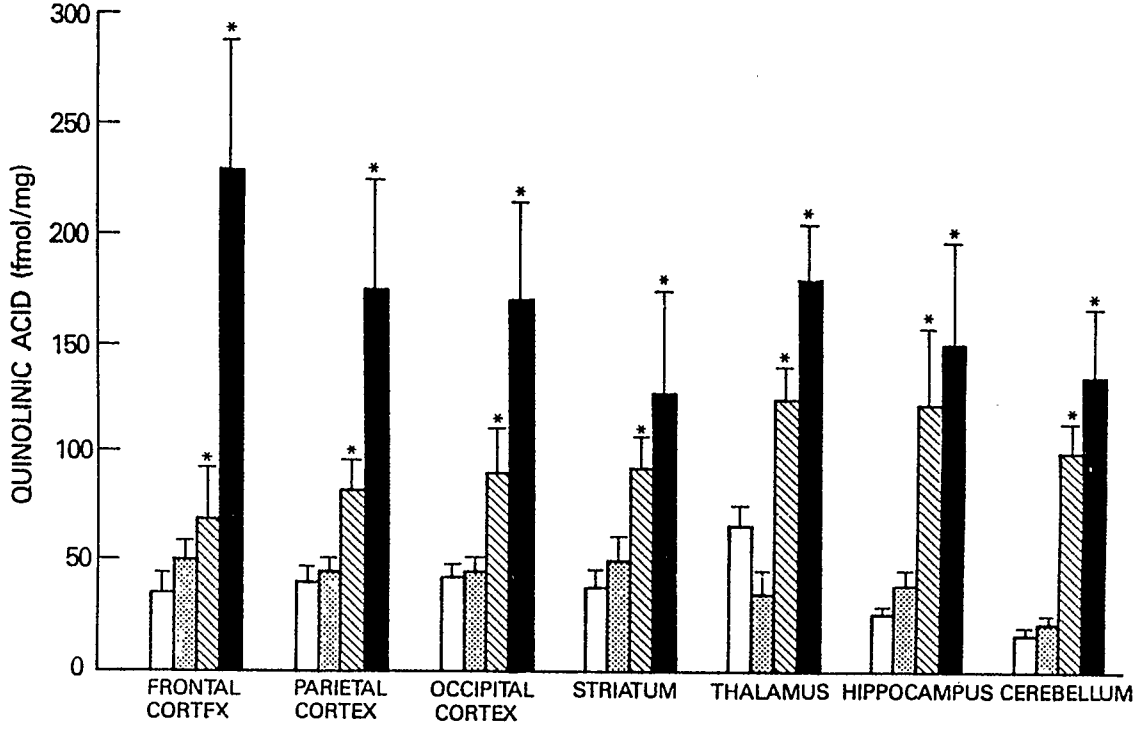

QUINOLINIC ACID (fmol/mg)
300
250
200
150
100
50
0
FRONTAL CORTEX
PARIETAL CORTEX
OCCIPITAL CORTEX
STRIATUM
THALAMUS
HIPPOCAMPUS
CEREBELLUM

Figure 14: Effects of profound hypoglycemia on regional brain L-TRP concentrations. Bar codes and comparisons as in Figure 13. * $p<0.01$. One-Way ANOVA with Dunnett's 't' test. In all brain regions, L-TRP concentrations increased during the recovery period only.

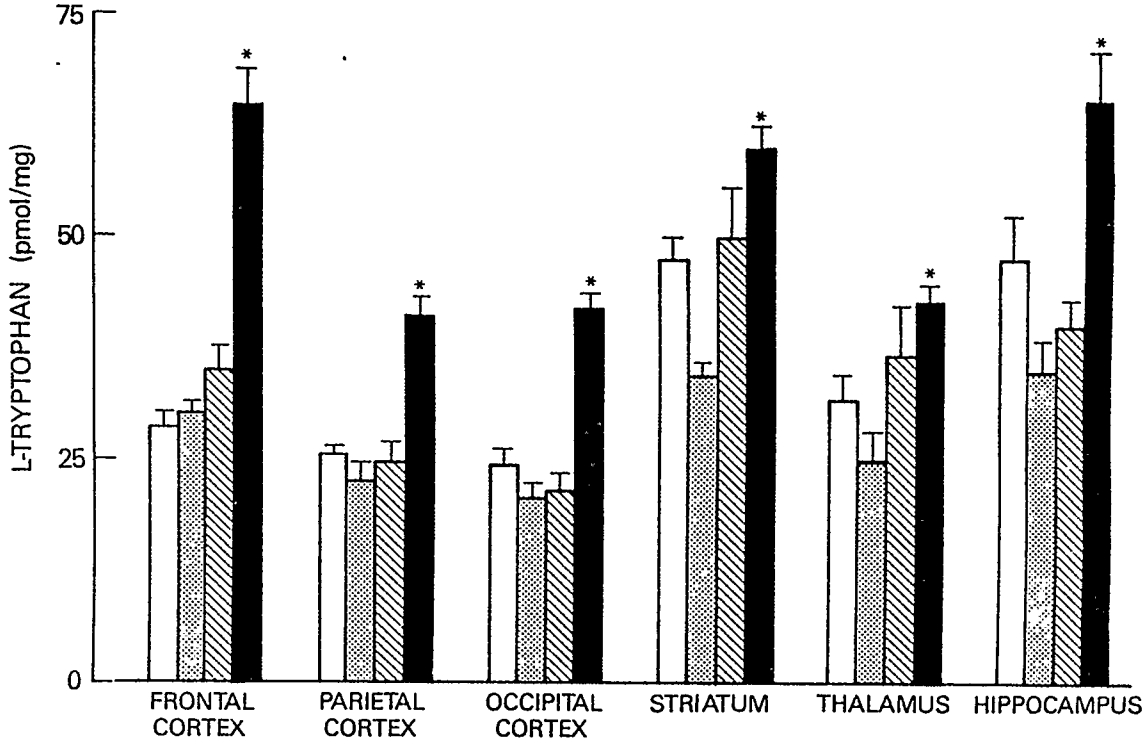

75
50
25
0
L-TRYPTOPHAN (pmol/mg)
*
FRONTAL CORTEX
PARIETAL CORTEX
OCCIPITAL CORTEX
STRIATUM
THALAMUS
HIPPOCAMPUS

Figure 15: Effects of profound hypoglycemia on regional 5-HT concentrations. Bar codes and comparisons as in Figure 13. * $p<0.01$. One-Way ANOVA with Dunnett's 't' test. In all brain regions, 5-HT concentrations decreased during hypoglycemia and increased towards normal values during recovery.

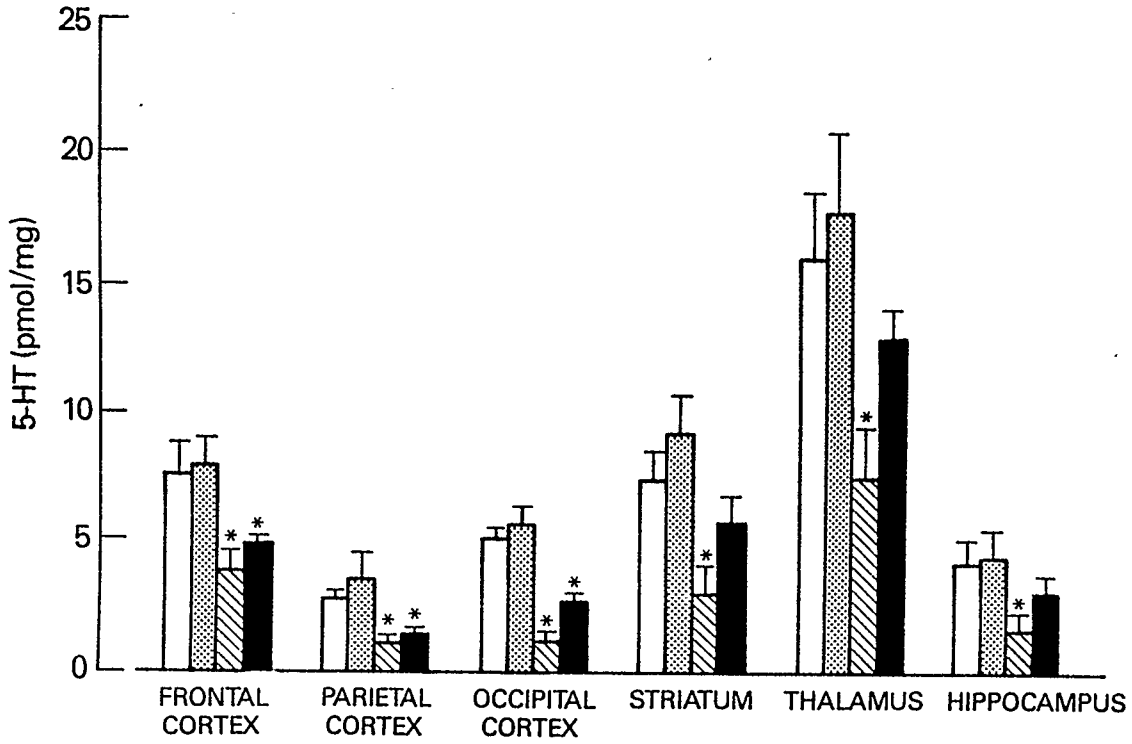

25
20
15
10
5
0
5-HT (pmol/mg)
*
FRONTAL CORTEX
PARIETAL CORTEX
OCCIPITAL CORTEX
STRIATUM
THALAMUS
HIPPOCAMPUS

Figure 16: Effects of profound hypoglycemia on regional brain 5-HIAA concentrations. Bar codes and comparisons as in Figure 13. * $p<0.01$. One-Way ANOVA with Dunnett's 't' test. In all brain regions, 5-HIAA concentrations were increased during both hypoglycemia and recovery.

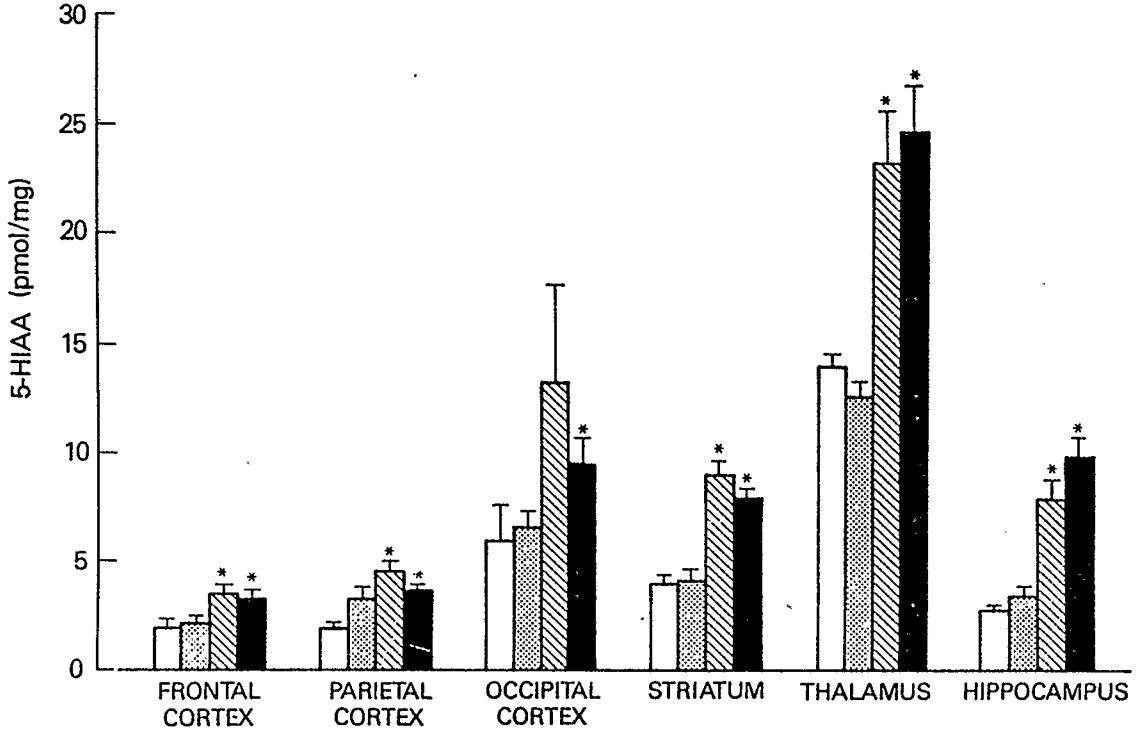

5-HIAA (pmol/mg)
30
25
20
15
10
5
0
FRONTAL CORTEX
PARIETAL CORTEX
OCCIPITAL CORTEX
STRIATUM
THALAMUS
HIPPOCAMPUS

Figure 17: Relative changes in QUIN, L-TRP, 5-HT, 5-HIAA, DA, DOPAC and HVA concentrations in striatum during hypoglycemia and recovery. Values for each species are expressed as percentage of control group values. Bar codes as in Figure 13. Pooled control and sham values were: QUIN, 39.9 ± 7.1 fmol/mg; L-TRP, 40.3 ± pmol/mg; 5-HT, 8.20 ± 0.62 pmol/mg; 5-HIAA, 3.99 ± 0.17 pmol/mg; DA, 79.5 ± 12.1 pmol/mg; DOPAC, 11.6 ± 6.7 pmol/mg and HVA, 8.50 ± 1.9 pmol/mg. * $p<0.01$.

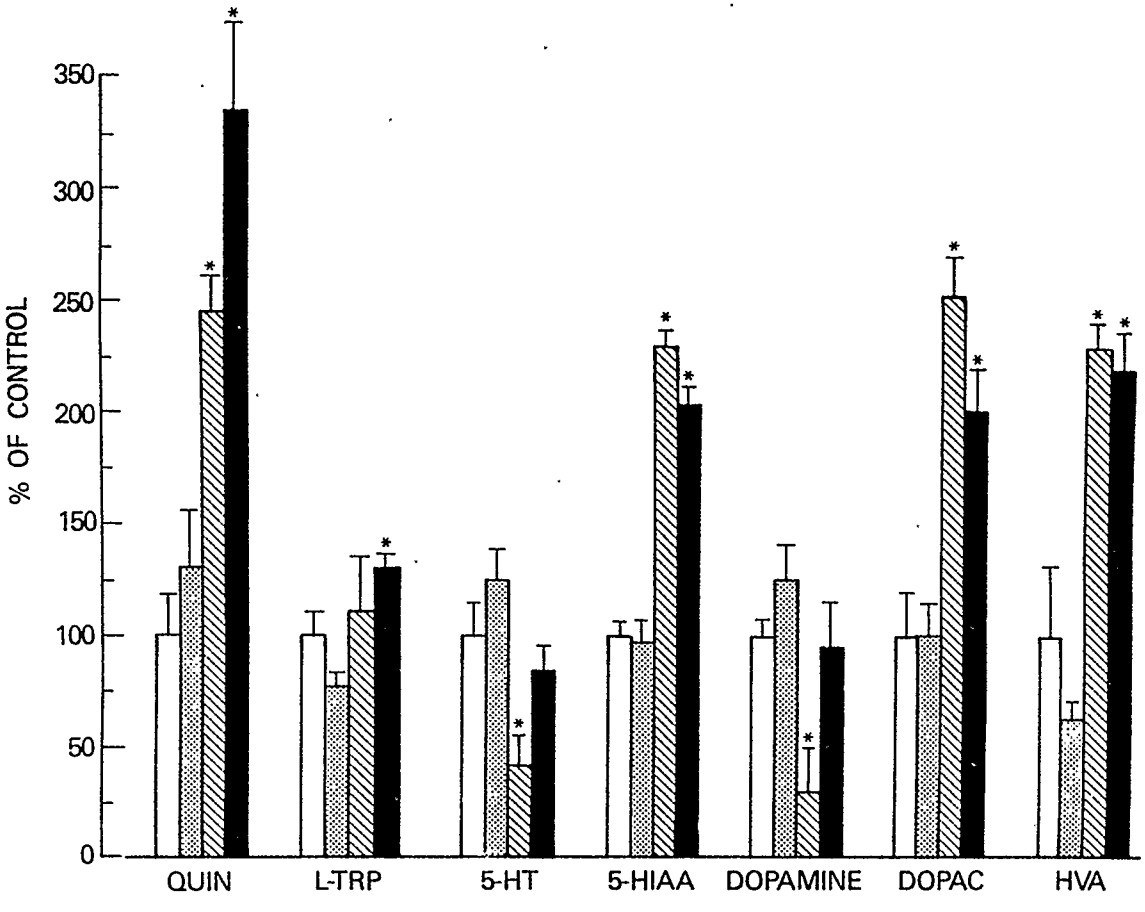

% OF CONTROL
350
300
250
200
150
100
50
0
QUIN
L-TRP
5-HT
5-HIAA
DOPAMINE
DOPAC
HVA

Figure 18: QUIN recovered from microdialysis probe inserted into the hippocampus. Microdialysis probes were inserted 24 h prior to injection of insulin. Rats were exposed to 2 h of insulin-induced hypoglycemia 40 min of isoelectricity and 2 h of normoglycemic recovery. Results are expressed as a percent of QUIN recovered during the control period. Legend as in Figure 12. The results indicate no changes in extracellular fluid QUIN concentrations at any time point examined.

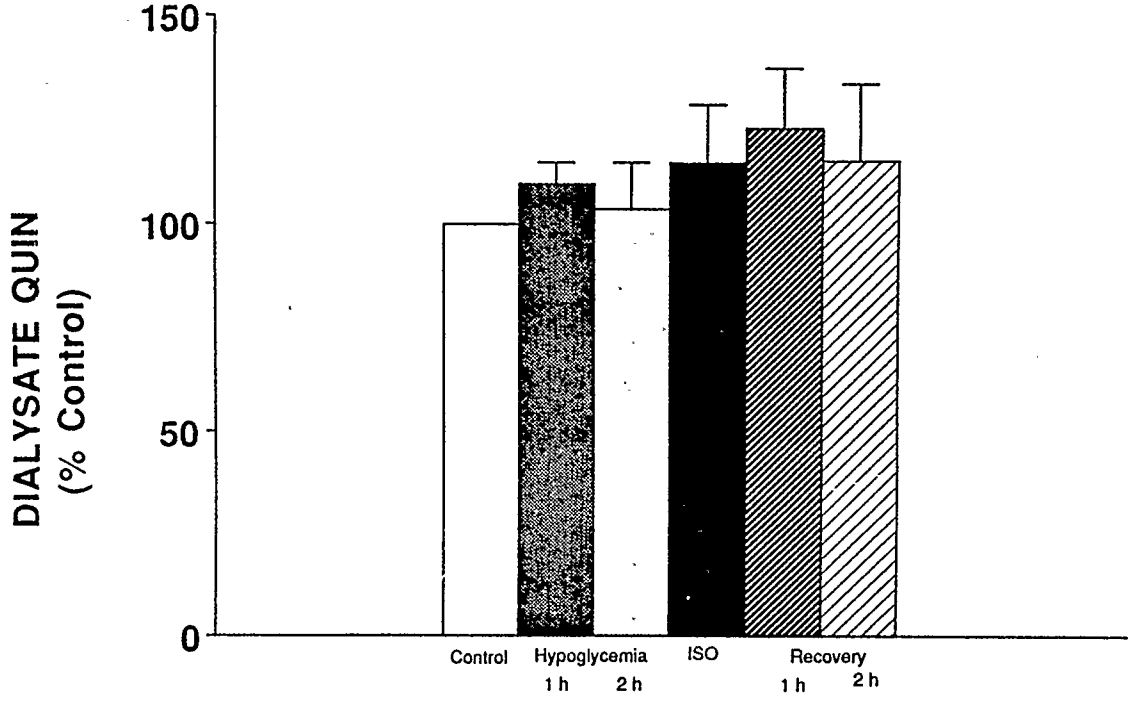

DIALYSATE QUIN
(% Control)
150
100
50
0
Control
Hypoglycemia
1 h
2 h
ISO
Recovery
1 h
2 h

Figure 19: Coronal section of a rat's brain. The arrows indicate the tract of the dialysis probe. Coordinates are: 2.0 mm lateral, 3.8 mm posterior to bregma and 3.5 mm below the skull surface.

Discussion

The present data show increases in regional brain QUIN concentrations during 40 min of cerebral isoelectricity which continued to increase during 1 h of normoglycemic recovery. Plasma QUIN concentrations also increased but reached peak values during hypoglycemia. However, there was no change in extracellular fluid QUIN concentrations as measured by in vivo microdialysis. These observations may indicate that the increases in brain tissue QUIN concentrations are restricted to the intracellular compartment of brain or are attributable to the presence of QUIN in the blood of these tissues. The increase in brain tissue QUIN concentrations (Figure 13) could be interpreted as being consistent with the notion that QUIN, via agonist effect on NMDA receptors, may be involved in the neuropathology of hypoglycemic brain damage. The type of neuronal damage produced by profound hypoglycemia is characteristic of excitotoxic damage. Excitotoxic lesions produced by QUIN have similar features to hypoglycemic brain damage and result when, for example, 12 nmol of QUIN is injected into the striatum [Schwarcz et al 1983]. Assuming a wet weight of 50 mg for a rat striatum, 12 nmol may be expected to produce peak QUIN concentrations of 240 μmol brain. This resultant concentration of QUIN far exceeds of the levels of QUIN observed in brain in hypoglycemia, which dilutes

the potential for QUIN to mediate hypoglycemic brain damage.

Access to NMDA receptors located on neuronal membranes by agonists is most likely from the extracellular space [Auer et al 1985c]. In this project an in vivo dialysis probe was implanted in the rat hippocampus to quantify changes in QUIN in the extracellular fluid during hypoglycemia. The hippocampus was chosen because of its susceptibility to hypoglycemic damage [Auer et al 1985b]. The microdialysate data indicate that extracellular fluid QUIN concentrations were not increased during either hypoglycemia or the recovery period (Figure 18). This observation does not support a direct role for increased extracellular fluid QUIN concentrations in early hypoglycemic brain damage. The fact that 3-hydroxyanthranilic added to the microdialysate perfusion medium increased QUIN synthesis indicates that the technique used would have been able to detect changes in extracellular fluid QUIN concentrations.

The concentration of plasma QUIN increased following administration of insulin and concomitant hypoglycemia. QUIN is formed predominantly in the liver from L-TRP via metabolism through the kynurenine pathway. The metabolic origin of QUIN in the brain is still unclear. However, because the blood brain barrier is relatively impermeable to QUIN [Foster et al 1984], it is likely that QUIN is actually synthesized in the brain. Assuming a plasma volume of 0.9 µl contained in

each aliquot of analysed brain tissue [Heyes and Markey 1988a], the contribution of plasma QUIN to measured regional brain QUIN content in control and sham rats was approximately 4% whereas in hypoglycemic rats the contribution was approximately 12%. Therefore, increases in brain tissue QUIN may be localized to the intracellular fluid compartment including astroglia where 3-hydroxyanthranilic-3,4-dioxygenase is localized [Okuno et al 1987]. Alternatively, it is possible that the changes in brain QUIN concentrations were actually due to the presence of increased amounts of QUIN in the blood compartment within the brain tissue sample [Heyes et al 1989]. The fact that extracellular fluid QUIN concentrations did not change during either hypoglycemia or recovery (Figure 18), despite increases in plasma QUIN concentrations (Figure 12), is consistent with a low permeability of the blood brain barrier to QUIN. It is surprising that although regional brain L-TRP concentrations were increased during the first hour of the recovery period (Figure 14), there were no changes in extracellular fluid QUIN concentrations as measured by the microdialysis probe. This observation may indicate that intracerebral L-TRP is a poor substrate for the synthesis of QUIN in brain or that increases in brain QUIN concentrations were restricted to the intracellar compartment.

During hypoglycemia, regional brain 5-HT concentrations

decreased at the same time as 5-HIAA concentrations increased This increase in 5-HIAA may reflect release of 5-HT from storage vesicles due to reduced energy stores to maintain the integrity of vesicular transport of the transmitter [Agardh et al 1979]. The same mechanism may account for the release of DA and concomitant increased synthesis of DOPAC and HVA in the striatum. The restoration of 5-HT concentrations during the recovery period may be related to increases in tryptophan hydroxylase activity in some brain regions [Agardh et al 1979] as well as increases in the concentrations of L-TRP (Figure 11) and conversion of 5-HT by mass action ratio effect [Fernstrom 1983].

Although insulin-induced hypoglycemia is associated with increased systemic synthesis of QUIN and perhaps an increase in QUIN concentrations within the intracellular compartment of brain, the concentrations of QUIN in the extracellular fluid space within brain is most likely unchanged. The project was originally designed to test the hypothesis that increased brain QUIN concentrations could perhaps play a role in the early induction of hypoglycemic brain damage. The data obtained from this project do not support a direct role for QUIN in the early excitotoxic lesions associated with profound insulin-induced hypoglycemia and cerebral isoelectricity.

Project 3: Hypotension and/or Hyperglycemia in the Post Isoelectricity (30 min) Recovery Period

This project was designed to test the hypothesis that hypotension and/or hyperglycemia during the recovery period after glucose administration can exacerbate cerebral lactic acidosis and can lead to brain infarction. Although lactic acid production cannot occur during profound hypoglycemia due to the lack of glucose, lactic acid production is possible during recovery when glucose is administered. The reason for suspecting a role for lactic acid arises from the finding of brain necrosis associated with focal lactic acidosis in numerous clinical conditions including Wernicke's encephalopathy [Hakim 1984], cerebral ischemia [Meyers 1979], mitochondrial encephalopathies [Pavlakis et al 1984, Riggs et al 1984], as well as substantia nigra necrosis in experimental epilepsy [Auer et al 1986b, Ingvar et al 1987].

The recovery model for insulin induced hypoglycemia was used in this experiment. Forty eight male Wistar rats weighing 250-300 g were divided into 4 groups of 12 rats, all of which underwent profound hypoglycemia under identical conditions. Only recovery parameters were varied between the groups. Of the 12 rats in each group, 6 were used for the neurochemical analysis of brain lactate, and 6 were allowed to survive one week for neuropathologic analysis. A further 4 groups, each consisting of 6 rats were used for brain lactate

analysis.

Group 1: Normoglycemia with Normotension (n=6)

The glucose infusion rate was decreased, and varied between 0.056-0.078 ml/min until the blood glucose levels were maintained between 5.6-10.5 mM. Blood pressure (BP) was allowed to vary between 100 and 120 mm Hg, after 30 min of EEG cerebral silence.

Group 2: Hyperglycemia with Normotension (n=6)

After 30 min of cerebral isoelectricity, the animals were given iv 25% glucose solution so as to maintain the blood glucose levels above 15 mM.

Group 3: Normoglycemia with Hypotension to 50 mm Hg (n=6)

After 30 min of cerebral isoelectricity, the animals were given 25% glucose solution so as to maintain the blood glucose levels between 5.6 and 10.5 mM while simultaneously lowering the blood pressure to 50 mm Hg via withdrawal of the blood through the prewarmed and heparinized central venous catheter and the administration of trimethaphan camphor sulphonate Arfonad (Hoffman LaRoche, Toronto, Ont.).

Group 4: Hyperglycemia (50 mm Hg) with Hypotension (n=6)

After 30 min of cerebral isoelectricity, the animals

were given 25% glucose solution so as to maintain the blood glucose levels above 15 mM, while simultaneously lowering the blood pressure to 50 mm Hg for 30 min with the administration of trimethaphan and the withdrawal of blood through the central venous catheter.

Group 5: Normoglycemia with Hypotension (35-40 mm Hg) (n=6)

After 30 min of cerebral isoelectricity, the animals were given 25% glucose solution so as to maintain blood glucose levels within a normoglycemic range, while simultaneously lowering the blood pressure between 40-35 mm Hg for 30 min with the administration of trimethaphan and the withdrawal of blood through the central venous catheter.

Group 6: Hyperglycemia with Hypotension (35-40 mm Hg) (n=6)

After 30 min of isoelectricity, the animals were given 25% glucose solution iv so as to maintain blood glucose levels above 15 mM, simultaneously lowering the blood pressure between 40-35 mm Hg for 30 min with the administration of trimethaphan and the withdrawal of blood through the central venous catheter.

Group 7: Sham Operated

The animals underwent a sham operation and then were sacrificed for brain lactate analysis.

Group 8: Controls (n=6)

The animals were fasted the night before and they were sacrificed out of their cages for brain lactate analysis.

The quantification of dead neurons was carried out by direct visual counting. Two forms of brain damage were graded: SNN, and pan-necrosis (death of neurons and glia). SNN was quantified according to the number of acidophilic neurons per section at standard coronal levels of the brain as previously described [Auer 1985a]. The number of hemispheres in each group showing a given degree of damage was compared.

Lactate Measurements

At the end of 30 min recovery, following 30 min of isoelectricity, the brains were frozen in situ by the transcalvarial technique [Pontén et al 1973]. The scalp was retracted and a truncated plastic funnel was fitted to the skull. Liquid nitrogen was poured into the funnel, producing a freezing front within the brain [Pontén et al 1973]. After 10 min, the brains were removed and a portion of the superolateral cortex was dissected and analyzed for lactate content using enzymatic fluorometric techniques. To measure cerebral cortical lactate content, the aliquot of cortex was weighed and mixed with an equal volume of 0.5 M perchloric acid ($HClO_4$). Following homogenization and centrifugation, a portion of the supernatant was reacted with NAD in the presence

of rabbit muscle lactic dehydrogenase (LDH) in a Dupont auto-analyser. Muscle lactic dehydrogenase (LDH) catalyses the oxidation of L-lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD). One mole of NAD is converted to one mole NADH for each mole (equivalent) of lactate present. The absorbance due to NADH is directly proportional to the lactate concentration and is measured spectrophotometrically using a double filter end point technique [Marbach and Weil 1967].

$$\text{L-lactate} + \text{NAD}^{+} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}$$

Hydrazine is used to trap the pyruvate (as a hydrazone) as it is formed, thus driving the reaction to completion.

The lactic acid analyses were carried out at the Biochemistry laboratories of Foothills Hospital, Calgary, Alberta.

Results

Animals in all groups remained in coma for 1-4 h following glucose administration, but could be extubated after approximately 4 h. There was no significant clinical difference between groups with respect to rate of awakening, or motor sluggishness on recovery.

Two animals intended to form part of group 4 had more profound and prolonged hypotension than planned. One rat

maintained a spontaneous blood pressure of 35 mm Hg for 30 min, in spite of complete reinfusion of the previously withdrawn blood. Blood pressure then gradually rose to 50 mm Hg at 45 min recovery, gradually recovering to normal over the subsequent half hour. A second rat developed hypotension to 40 mm Hg for 25-30 min, with gradual recovery of blood pressure to normal over the ensuing hour. Because these animals showed cortical infarction, an additional two groups of six animals were designed for neurochemical measurements of brain lactic acid values at these blood pressures.

The physiologic measures for the experiment are provided in Table 2. Quantitated neuronal necrosis in the cerebral cortex, caudate nucleus, dentate gyrus and CA1 region of the hippocampus showed no difference among the 4 groups. The quantification of necrotic neurons was carried out by direct visual counting. Two forms of brain damage were graded: SNN (Figure 20) and pan-necrosis (death of neurons and glia, Figures 21, 22). SNN was quantified according to the number of acidophilic neurons per section at standard coronal levels of the brain as previously described [Auer et al 1984b]. Such acidophilic neurons have been previously shown to be necrotic in electron microscopic, temporal studies [Auer et al 1985b]. The number of hemispheres in each group showing a given degree of damage was compared (Table 3). Three of the rats which underwent severe hypotension (< 50 mm Hg) after hypo-

glycemia showed cortical infarctions, 2 in the cortex and 1 in the caudate. These animals were clinically indistinguishable from the group 4 animals.

Control brain lactate levels in the cerebral cortex were 2.2 ± 0.2 μmol/g. Animals which underwent 30 min of cerebral isoelectricity with normoglycemia and normotension in the recovery period showed brain lactate levels of 6.6 ± 1.1 μmol/g wet tissue. The group which underwent 30 min of cerebral silence and recovery was hyperglycemic and normotensive, had brain lactate measurements of 4.6 ± 0.4 μmol/g. The sham operated group had a brain lactate level of 9.1 ± 2.0 μmol/g wet tissue. The group with the combined hyperglycemia and hypotension to 50 mm Hg in the recovery period showed lactate values of 10.1 ± 0.7 μmol/g wet tissue in the cerebral cortex (Figure 23). The group with the combined normoglycemia and hypotension to 50 mm Hg in the recovery period had brain lactate measurements of 7.3 ± 0.7 μmol/g. The animals with hypotension to 35 mm Hg and hyperglycemia, showed a marked rise in lactate to 15.4 ± 1.8 μmol/g. The normoglycemic combined hypotensive (35-40 mm Hg) group showed cortex lactate values of 12.0 ± 3.0 μmol/g.

From the data, it was evident that the highest brain lactate measurements were to be found in the hyperglycemic and severe hypotensive group. The infarcts which were observed in this experiment (2 cortices and 1 caudate) were all

found in the above mentioned group. All other groups showed selective neuronal necrosis characteristic of excitotoxic brain damage.

Table 2. Physiologic measures for the experiment. All values are mean ± S.E.M. One-Way ANOVA with Dunnett's 't' test * $p<0.05$.

Table 2:

Physiologic Measures during Insulin Induced Hypoglycemia and in the Recovery Period Following Glucose Administration

	Control	Hyperglycemia	Hypotension	Hyperglycemia with Hypotension
MABP (iso)	117.3 ± 5.8	125.7 ± 6.5	114.0 ± 0.0	*105.3 ± 8.9
MABP(post)	115.3 ± 7.0	129.5 ± 3.0	54.0 ± 1.0	54.0 ± 1.0
Body Temperature (°C)	37.5 ± 0.0	37.5 ± 0.1	37.6 ± 0.1	37.5 ± 0.1
Blood Glucose (iso)	0.9 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.0
Blood Glucose (post)	9.2 ± 1.7	*19.5 ± 0.6	6.6 ± 1.0	*19.0 ± 1.0
pH(iso)	7.339 ± 0.020	7.367 ± 0.022	7.356 ± 0.022	7.348 ± 0.017
pH(post)	7.376 ± 0.013	7.335 ± 0.019	7.324 ± 0.022	7.315 ± 0.026
pCO_2(iso)	31.9 ± 2.2	32.6 ± 1.7	*40.1 ± 2.6	32.5 ± 1.8
pCO_2(post)	38.5 ± 2.1	36.8 ± 2.3	36.8 ± 1.7	38.8 ± 2.3
pO_2(iso)	110.5 ± 4.1	115.2 ± 3.3	*139.2 ± 17.1	118.8 ± 11.5
pO_2(post)	109.7 ± 2.4	116.6 ± 4.2	113.6 ± 6.6	*120.7 ± 4.7

Figure 20: Selective neuronal necrosis (SNN). Dead neurons exhibit an intense affinity for acid stains, appearing black and punctate in the photomicrograph, taken from a rat with 30 min of electrocerebral (EEG) silence due to profound hypoglycemia (35-40 mm Hg), followed by one week recovery. 2% phloxine, 0.1% cresyl violet. Bar = 500μm.

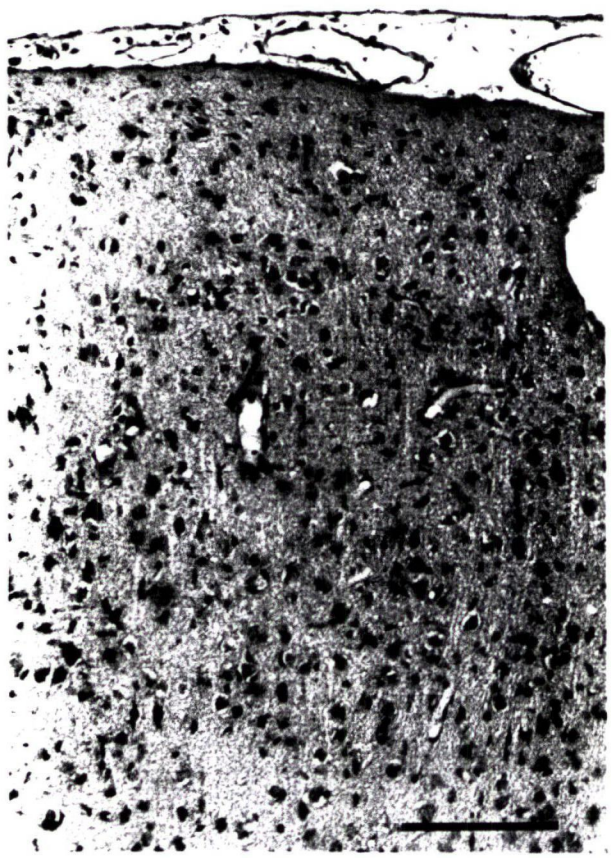


Figure 21: Pan-necrosis shows cavitation of the neuropil, with a dense infiltrate of macrophages. Rats with hypotension to 35-40 mm Hg in the recovery period showed cortical pan-necrosis. Rat cerebral cortex, 2% phloxine, 0.1% cresyl violet. Bar = 500μm.

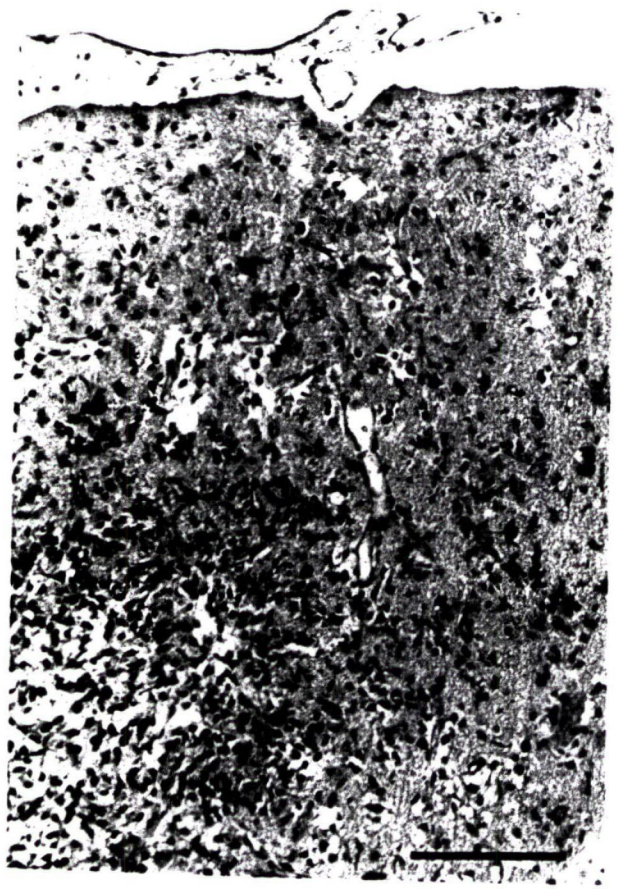


Figure 22: An infarct in the caudate of a rat which underwent 30 min of profound hypotension in conjunction with hyperglycemia in the recovery period. Macrophage infiltration is seen circumscribing several blood vessels. 2% phloxine, 0.1% cresyl violet. Bar = 50μm.

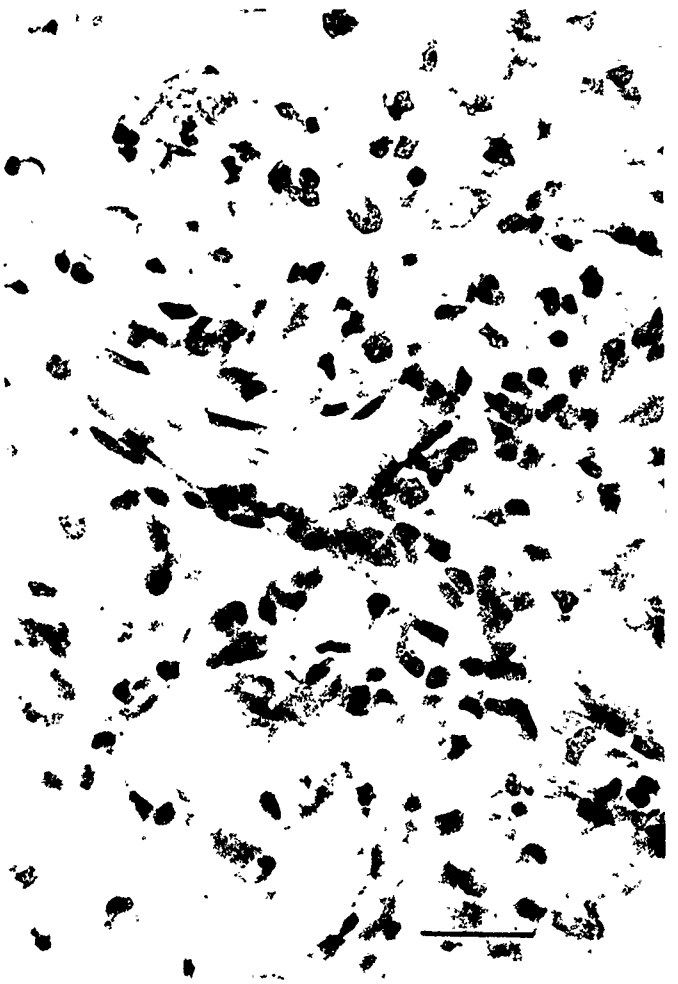


Figure 23: Blood glucose, brain lactate, and blood pressure in the recovery period after hypoglycemia. Normal rats showed a brain lactate level of 2.2 µmol (not shown), but brain lactate levels increased in the recovery, probably due to relative hypoperfusion (Agardh et al 1980a). Lactate accumulation is exacerbated by a combination of hyperglycemia and hypotension to 50 mm Hg, and is further exacerbated to the range of 15 µmol by hypotension to 35 mm Hg.

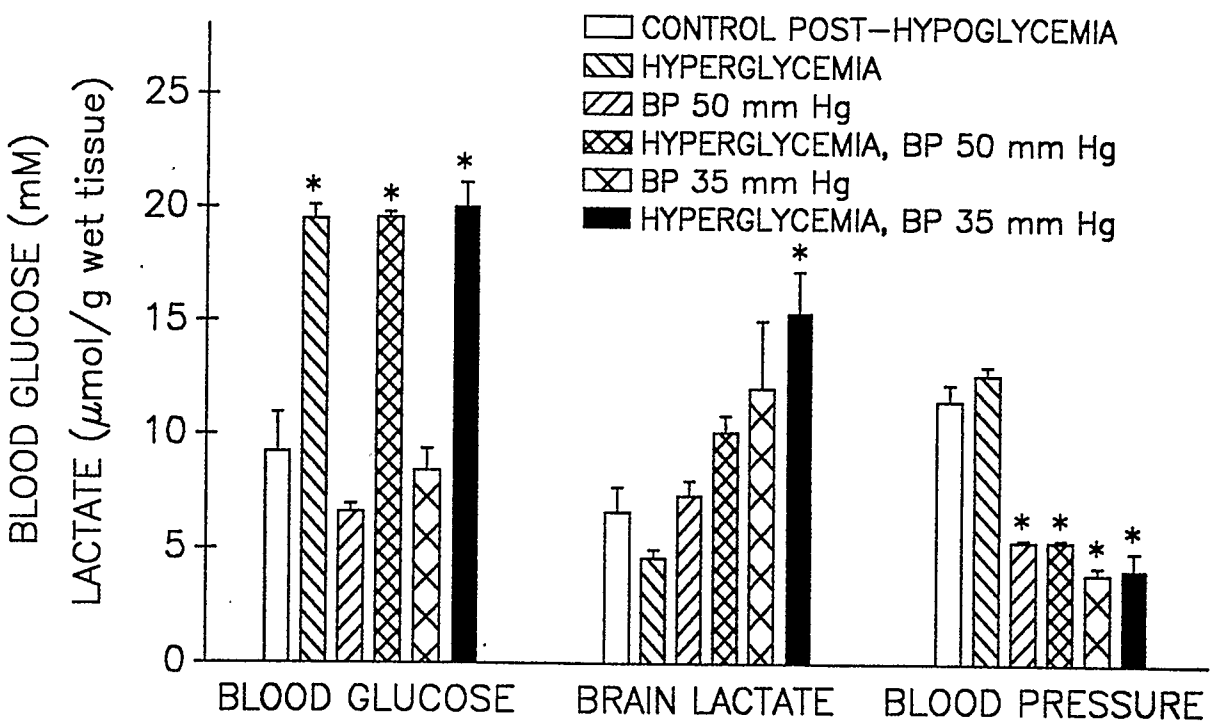

CONTROL POST–HYPOGLYCEMIA
HYPERGLYCEMIA
BP 50 mm Hg
HYPERGLYCEMIA, BP 50 mm Hg
BP 35 mm Hg
HYPERGLYCEMIA, BP 35 mm Hg
BLOOD GLUCOSE (mM)
LACTATE (μmol/g wet tissue)
0
5
10
15
20
25
*
BLOOD GLUCOSE
BRAIN LACTATE
BLOOD PRESSURE

Table 3. Quantitated neuronal necrosis in the cerebral cortex caudate nucleus, dentate gyrus and CA1 region of the hippocampus showed no difference among the 4 groups. Kruskal-Wallis ANOVA with tied ranks.

Table 3:

Selective Neuronal Necrosis in Cortex, Caudate and Hippocampus

	Control	Hyperglycemia	Hypotension	Hyperglycemia with Hypotension
CORTEX				
<10 cells/section	2	6	8	6
11-100 cells/section	6	0	1	4
101-1000 cell/section	4	2	1	0
>1000 cells/section	0	0	2	0
CAUDATE				
<10% necrotic cells	0	0	1	0
10-50% necrotic cells	2	4	6	7
>50% necrotic cells	10	8	4	5
DENTATE				
<10% necrotic cells	4	5	4	4
10-50% necrotic cells	8	7	6	2
>50% necrotic cells	0	0	2	4
CA1				
<10% necrotic cells	2	9	8	6
10-50% necrotic cells	10	3	0	5
>50% necrotic cells	0	0	2	1

Discussion

This study showed that if hyperglycemia and hypotension are present in the recovery period after profound hypoglycemia, to a degree giving rise to cortical lactate levels of at least 15.4 ± 1.8 μmol/g wet tissue, infarction or pan-necrosis in the cortex and caudate may occur. The pathogenesis of pan-necrosis, where by glia and other non-neuronal cell types in the brain are killed, is believed to involve an accumulation of tissue lactic acid in ischemia and epilepsy [Auer and Siesjö, 1988].

Support for a role for lactic acid in the pathogenesis of pan-necrosis within the brain comes from the demonstration of focal acidosis in the vulnerable areas in Wernicke's disease [Hakim 1984], focal lactate accumulation preceding necrosis in the substantia nigra of the rat in epilepsy [Ingvar et al 1987] and lastly CSF and brain lactate accumulations in mitochondrial encephalopathies [Pavlakis et al 1984, Kuriyama et al 1984]. Histologically they resemble infarcts in that they demonstrate liquefactive necrosis and removal of the neuropil by macrophages.

There are several reasons for suspecting that lactate-related infarcts might occur due to events in the recovery period after profound hypoglycemia. Firstly, although CBF is increased during hypoglycemia, there is marked hypoperfusion

in the recovery period [Abdul-Rahman et al 1980]. In fact, the rebound period of hypertension often seen in the recovery period after profound hypoglycemia is insufficient to raise CBF above one third to one half the normal blood flow rates in most regions of the cerebral cortex [Abdul-Rahman et al 1980]. At similar recovery times, the CMR_{glu} shows a better recovery in the same cortical regions. If glucose is administered in excess at this time, a flux through the glycolytic pathway might be produced which could exceed the capacity of oxidative metabolism to handle the resultant production of lactate/pyruvate. Lactic acid accumulation within the brain was shown to be augmented by posthypoglycemic hypotension in the present study. However, histologic infarction was not seen unless lactic acid accumulated to the range of 15.4 ± 1.8 μmol/g.

The underlying mechanisms of the histotoxicity of lactic acid to brain tissue probably involves a drop in pH. Indeed, when lactic acid is injected directly into the brain, cavitating lesions result when the pH of the injectate approaches 4.5 [Petito et al 1987]. At a pH of 7.4 the lactate injection does not cause any observed lesions. The hydrogen ion would thus seem to be the critical determinant of lactate histotoxicity, and has been shown to cause brain necrosis at concentrations reached during ischemia [Kraig et al 1987]. To avoid a potentially damaging cortical lactic acidosis and cerebral

infarction in clinical situations, it would seem prudent to prevent hyperglycemia and hypotension when recovering patients who have had profound hypoglycemia.

CONCLUSIONS

In this thesis, hypoglycemic brain damage was investigated with neurochemical and histologic techniques. In the first project of the thesis an attempt was made to mitigate hypoglycemic brain damage with the NMDA non-competitive antagonist MK-801. The results generally showed protection in the susceptible brain areas. The most pronounced reduction in neuronal necrosis was observed in the caudate. However, in all brain areas studied, damage was attenuated with the highest dose of the antagonist. In the dentate gyrus of the hippocampus and caudate nucleus, mitigation of brain damage was more effective with the 1.5 than with the 2.8 mg/kg dose of MK-801. Several possible reasons could explain these results. The MK-801 was injected during the delta stage of cerebral activity but the time between the delta stage of EEG and the resulting isoelectricity usually varied for each rat. Therefore, there was always the possibility that in some animals there was not sufficient time for the MK-801 to pass through the blood brain barrier and bind to the NMDA recep-

tors. Another problem could have been due to the fact that surgery was not performed within a consistent time frame; some rats were operated in the morning, others in the afternoon and still others late in the evening. Because biochemical and circadian biorhythms in nocturnal animals such as rats have different physiological effects, consistency in the time of surgery would have been preferable.

In humans, hypoglycemic brain damage shows a pattern of damage which parallels that of experimental animals [Auer et al 1989]. Profound hypoglycemia leads to a clinical syndrome very similar to that following hypoxia-ischemia with total loss of higher cortical function. Usually hypoglycemic comatose patients show a slowing of their EEG and a lack of sensory evoked responses. These observations indicate widespread neuronal damage [Agardh et al 1983]. The importance of mitigation of hypoglycemic brain damage is evident from the above discussion.

There is an ongoing controversy concerning the mechanism(s) by which MK-801 mitigates brain damage. Pulsinelli and his associates have provided as yet unpublished evidence that the protective effect of MK-801 depends on its hypothermia inducing properties during an ischemic insult. The results of the first project of this thesis tend not to support such a hypothermic mechanism. During hypoglycemic isoelectricity the brain is hyperperfused [Abdul-Rahman et al 1980],

while hypoperfusion occurs after glucose administration. Since the body temperature was constantly monitored, and the brain was being hyperperfused during the period of iso-electricity, its temperature was likely to be close to body temperature. The mechanism by which MK-801 mitigates hypoglycemic brain injury probably involves NMDA receptor blockade in several pathologic states. The efficacy in mitigating hypoglycemic and ischemic brain damage has been well documented [McDonald et al 1987, Gill et al 1988, Park et al 1988, Kochhar et al 1988, Olney et al 1989]. In ischemic brain damage, there is a delayed hyperexcitability in the CA1 sector of the hippocampus and in the cortex [Suzuki et al 1983b] of the gerbil after an ischemic insult. Kirino also observed histologically that in ischemia there is an observed delayed neuronal necrosis [Kirino 1982] after an ischemic insult. This delayed neuronal necrosis implies a temporal therapeutic window during which pharmacological intervention is possible. In hypoglycemic brain damage, neurons which are destined for necrosis usually die within the first 6-8 h after the insult; thus in a clinical setting, pharmacological intervention will have to occur as soon as possible. Further studies based on the conclusions of this project seem warranted. For example, it would be clinically useful to know whether MK-801 is still useful in preventing hypoglycemic neuronal necrosis when administered at the onset of cerebral

isoelectricity. The results of such a study would be of clinical relevance if NMDA antagonists such as MK-801 are introduced into clinical practice.

The results of the second project of the thesis show that the probability of QUIN being involved in the pathogenesis of hypoglycemic brain damage is remote. Since the concentration of QUIN, as monitored by microdialysis, did not rise, whereas an increase in brain and plasma QUIN was seen, the apparent increase in brain QUIN can be attributed to an increase in QUIN in the contained blood in the vessels.

The third part of the thesis leads to the conclusion that only profound hypotension in conjunction with hyperglycemia leads to a risk of pan-necrosis. This damage is characteristic in its morphology and different from selective neuronal necrosis. Although the measured increase in lactic acid associated with these infarcts does not prove that lactate is involved inn their pathogenesis, the levels of lactate seen, 15-17 μmol in the tissue, are comparable to those demonstrated to be associated with ischemic infarction and necrosis of the substantia nigra in epilepsy [Ingvar et al 1987].

A blood pressure of 35-40 mm Hg can, and probably does leave the brain ischemic. Although the pan-necrosis observed was only in the group which had the combined hyperglycemia-hypotension recovery parameters, the possibility cannot be excluded that the pan-necrosis was not also a function of the

extreme hypotension. Cerebral blood flow experiments are warranted to further pursue the matter as to the role of ischemia in the pathogenesis of pan-necrosis. The findings suggest it would be prudent to avoid hypotension in the clinical recovery of hypoglycemic patients.

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