Novel Hypoglycemic Injury Mechanism: N-Methyl-D-Aspartate Receptor–Mediated White Matter Damage

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Objective: Hypoglycemia is a common adverse event and can injure central nervous system (CNS) white matter (WM). We determined whether glutamate receptors were involved in hypoglycemic WM injury.

Methods: Mouse optic nerves (MON), CNS WM tracts, were maintained at 37°C with oxygenated artificial cerebrospinal fluid (ACSF) containing 10mM glucose. Aglycemia was produced by switching to 0 glucose ACSF. Supramaximal compound action potentials (CAPs) were elicited using suction electrodes, and axon function was quantified as the area under the CAP. Amino acid release was measured using high-performance liquid chromatography. Extracellular lactate concentration ([lactate]o) was measured using an enzyme electrode.

Results: About 50% of MON axons were injured after 60 minutes of aglycemia (90% after 90 minutes); injury extent was not affected by animal age. Blockade of N-methyl-D-aspartate (NMDA)-type glutamate receptors improved recovery after 90 minutes of aglycemia by 250%. Aglycemic injury was increased by reducing [Mg2+]o or increasing [glycine]o, and decreased by lowering pHo, expected results for NMDA receptor-mediated injury. pHo increased during aglycemia due to a drop in [lactate]o. Aglycemic injury was dramatically reduced in the absence of [Ca2+]o. Extracellular aspartate, a selective NMDA receptor agonist, increased during aglycemia ([glutamate]o fell).

Interpretation: Aglycemia injured WM by a unique excitotoxic mechanism involving NMDA receptors (located primarily on oligodendrocytes). During WM aglycemia, the selective NMDA agonist aspartate is released, probably from astrocytes. Injury is mediated by Ca2+ influx through aspartate-activated NMDA receptors made permeable by an accompanying alkaline shift in pHo caused by a fall in [lactate]o. These insights have important clinical implications.

Hypoglycemia continues to be an important and common adverse clinical event in patients with diabetes, and more rarely with other conditions.1–4 The central nervous system (CNS) is extremely vulnerable to dysfunction and injury with hypoglycemia.1,5,6 In addition, there is compelling evidence that CNS dysfunction can develop slowly over years in patients with well-controlled diabetes7,8 (see also Won et al9). Cognitive dysfunction has been linked to hypoglycemic episodes7 and to chronic hyperglycemia associated with microvascular disease.8 For these reasons, it is important to understand how hypoglycemia produces irreversible CNS injury. This knowledge will provide a basis for development of effective therapies to minimize the extent of damage and improve clinical outcome.

Recent observations have drawn attention to selective white matter (WM) damage as a consequence of protracted hypoglycemia10,11; in fact, WM can be the predominant site of injury.10,11 However, experimental studies on the mechanisms of hypoglycemic injury have used primarily rodents. As discussed elsewhere, humans have roughly 4- to 5-fold more WM than rodents, a critical difference because WM and gray matter (GM) are vastly different tissues with unique mechanisms of injury.12,13

Fortunately, validated and practical models of CNS WM injury are now available.14–17 Initial studies using...
the acutely isolated mouse optic nerve (MON), a myelinated WM tract, found that 60 minutes of aglycemia produced substantial irreversible injury. The mechanism of injury was found to be Ca\(^{2+}\)-dependent and to involve activation of L-type Ca\(^{2+}\) channels and reverse Na\(^+\)/Ca\(^{2+}\) exchange.\(^6\) These two pathways mediated toxic Ca\(^{2+}\) influx, contributing to permanent loss of axonal excitability.\(^6\) Subsequently, another form of energy disruption in WM, ischemia, was found to produce injury mediated by excessive activation of excitatory glutamate receptors. This pathogenesis, called excitotoxicity, was first defined in CNS GM.\(^{18}\) The characteristics of excitotoxicity in WM are distinct from GM; in other words, the toxic glutamate receptor activation seen in WM during ischemia is a special form of excitotoxicity.\(^{12,17,19–21}\) Although both N-methyl-D-aspartate (NMDA) and non-NMDA-type glutamate receptors (NMDARs) are found in WM, only non-NMDARs appear to participate in irreversible functional ischemic (i.e., oxygen and glucose deprivation or OGD) WM injury\(^{15,19,21–23}\) (but see Bakiri et al\(^ {24}\)). This was somewhat unexpected, because NMDARs are clearly expressed by myelinating oligodendrocytes,\(^ {19,25}\) and these cells are unquestionably injured during energy deprivation.\(^ {19,20,22,23,26}\) Curiously, however, NMDAR blockade reduced the extent of morphological myelin injury after chemical ischemia, but failed to protect the underlying axons.\(^ {25}\)

We sought to determine whether excitotoxicity might also be involved in the WM injury seen with aglycemia. We found that excitotoxicity contributed importantly to this special type of irreversible WM injury, but unlike the situation with OGD, in aglycemia NMDARs were prominently involved, in a Ca\(^{2+}\)-dependent manner. Our results also provided a plausible explanation for why NMDARs were involved in aglycemia but not in ischemia. Finally, we showed that WM aglycemic injury arose from NMDARs activated by aspartate, not glutamate. These findings raise important questions about how to optimally manage the clinical situation of severe hypoglycemic brain dysfunction.

Materials and Methods

All experiments were done in accordance with the University of Washington Institutional Animal Care and Use Committee.

Electrophysiology

MONs were acutely obtained from C57BL/6 mice that varied in age from 1 to 24 months of age; most experiments were done on 3-month-old mice. As previously described,\(^ {27}\) mice were deeply anesthetized with CO\(_2\) and then decapitated. Optic nerves were dissected free and cut at the optic chiasm and behind the orbit. The optic nerves were freed from their dural sheaths and placed in an interface perfusion chamber (Medical Systems, Greenvale, NY) and maintained at 37°C. MONs were superfused with artificial cerebrospinal fluid (ACSF) containing (in millimoles per liter): 125 NaCl, 3.0 KCl, 2.0 CaCl\(_2\), 2.0 MgSO\(_4\), 7H\(_2\)O, 1.25 Na\(_2\)HPO\(_4\), 26 NaHCO\(_3\), and 10 glucose. The ACSF was bubbled with an O\(_2\)-free gas mixture (95% N\(_2\)/5% CO\(_2\)) to maintain pH at 7.45. A humidified gas mixture of 95% O\(_2)/5% CO\(_2\) continuously aerated the chamber and adequately supplied oxygen to the tissue.\(^ {28}\) Two sets of suction electrodes were placed in the bath to allow recording from 2 optic nerves at the same time. Suction electrodes backfilled with the appropriate ACSF were used for stimulating and recording. The stimulating electrode was attached to the proximal end of the nerve, whereas the distal end was attached to a second electrode to record the compound action potential (CAP), ensuring orthodromic stimulation. Stimulus pulse (30-microsecond duration) strength (Isostim 520; WPI, Sarasota, FL) was adjusted to evoke the maximum CAP and then increased another 25% (ie, supramaximal stimulation).\(^ {14}\) During an experiment, the supramaximal CAP was elicited every 30 seconds. The recording electrode was connected to an amplifier (Model SR560; Stanford Research Systems, Sunnyvale, CA), and the signal was amplified 500\(\times\), filtered at 30kHz and acquired at 20kHz.

Nerves were allowed to equilibrate for at least 30 minutes before recording commenced. In glucose deprivation experiments, the solution in the stimulating and recording electrodes was switched to glucose-free ACSF (ie, aglycemia). Osmotic compensation was achieved by adding 10mmol/l sucrose. Ca\(^{2+}\)-free ACSF was made by omitting CaCl\(_2\) and adding 0.5mmol/l ethylene glycol-bis (\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) with equimolar MgCl\(_2\). Zero-Mg\(^{2+}\) ACSF was made by omitting MgSO\(_4\) and replacing it with equimolar Na\(_2\)SO\(_4\). In a few experiments, both oxygen and glucose were deleted to create an ischemia-like condition, also referred to as OGD.

Bath pH was changed from 7.45 to 7.00 by reducing the \([\text{HCO}_3^-]\) using the following ACSF recipe (in millimole per liter): 141 NaCl, 3.0 KCl, 2.0 CaCl\(_2\), 2.0 MgSO\(_4\), 7H\(_2\)O, 1.25 Na\(_2\)HPO\(_4\), 10 NaHCO\(_3\), and 10 glucose. All changes in ACSF were introduced 15 minutes before the insult and continued until 15 minutes after terminating the insult (eg, Baltan et al\(^ {15}\)).

Pharmacological agents were applied for 15 minutes before the insult and continued until 15 minutes after terminating the insult. The following agents were purchased from Tocris (Ellisville, MO): 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; 30\(\mu\)M; NMDAR blocker; dissolved in DMSO as 15mM stock solution).\(^ {29}\) Glycine, \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate channel blocker; dissolved in dimethylsulfoxide (DMSO) as 30mM stock solution), 7-chlorokynurenic acid (7-CKA; 50\(\mu\)M; glycine binding site blocker; dissolved in DMSO as 50mM stock solution), and (5S,10R)- (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepte-n-5,10-imine maleate (MK801; 15\(\mu\)M; NMDAR blocker; dissolved in DMSO as 15mM stock solution).

Lactate Biosensor

Lactate and null biosensors were purchased from Sarissa Biomedica (Coventry, UK). In these experiments, however, the
lactate signals were sufficiently large that subtraction of the null signal did not meaningfully alter the lactate signal amplitude. The lactate biosensors (25 μm in diameter and 500 μm in length) were pressed against the pial–glial membrane of the MON. Experimental recordings began after an equilibration period of 30 to 60 minutes. At the beginning and end of all experiments, lactate biosensors were calibrated using lactate concentrations of 10, 100, and 1,000 μM. Results were considered valid only if the before and after calibrations deviated by no more than 5%.

**pH-Sensitive Microelectrodes**

Ion-sensitive microelectrodes for extracellular pH (pH₄) measurements were made according to the method of Borrelli et al. with slight modifications. Briefly, double-barrel microelectrodes were pulled and beveled to a tip diameter of 2 to 5 μm. The ion-sensitive barrel was backfilled with a short column of H⁺ sensitive sensor (Fluka pH ionophore; Sigma-Aldrich, St Louis, MO). The indifferent barrel was backfilled with 140 mM NaCl + 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid adjusted to pH 7.0. Only electrodes that showed near-Nernstian responses to 10-fold changes to [H⁺] were used in experiments.

**Data Analysis**

Optic nerve function was quantitatively determined by integrating the area under the CAP. Data were acquired online (Digitata 1440A; Molecular Devices, Sunnyvale, CA) using proprietary software (Clampex, Molecular Devices). CAP area was calculated using pClamp (Molecular Devices) and was normalized by averaging the baseline CAP area over a period of 15 minutes, and setting this value to 1.0. The normalized CAP area at any time is proportional to the relative number of functioning axons.

**Glutamate, Aspartate, and Glycine Measurements**

Amino acid (ie, glutamate, aspartate, glycine, etc) release from MON was determined by first collecting the superfusate during 60 or 90 minutes of aglycemia (or OGD in a few experiments) and then subjecting these samples to quantitative amino acid measurement using high-performance liquid chromatography (HPLC). Experiments were designed to monitor amino acid release simultaneously from 1 pair of MONs (this technique can be modified to enhance sensitivity by using 5 pairs of MONs measured at one time). Briefly, amino acids were pre-column derivatized with o-phthalaldehyde (Sigma, St Louis, MO), separated, and measured using standard techniques. Samples of extracellular perfusion fluid were collected continuously such that every vial contained either 1 or 2 minutes of superfusate. Glutamate content was measured in every, or every other, vial (ie, every 1 or 2 minutes). Collected samples were centrifuged at 16,000 × g for 3 minutes, and supernatants were transferred for HPLC analysis. Glutamate measurements, normalized to baseline glutamate release, were made from MONs treated identically to those studied electrophysiologically, and the results were plotted against time. The rate and release pattern of glutamate was monitored for 30 minutes before injury (60 minutes or 90 minutes aglycemia, or 60 minutes OGD), and was continued for at least 30 minutes after the end of the insult. Actual amounts of glutamate measured in millimoles were determined by comparing the experimental measurements to standard samples.

Intracellular amino acid content of cells within the MON was determined using a slightly modified established technique. Briefly, pairs of MONs were sonicated in 0.2 ml of homogenization solution (distilled H₂O supplemented with 0.4 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid). The homogenate was diluted 10-fold in homogenization solution prior to protein measurement and HPLC measurement of amino acids. Aliquots for HPLC measurement of amino acids were then acidified by HCl and centrifuged. The supernatant was harvested and neutralized by NaOH prior to using HPLC to measure amino acid concentrations. Protein content was measured in identical aliquots using Bio-Rad (Hercules, CA) protein assay reagent.

**Results**

**Aglycemia Caused Duration-Dependent WM Injury**

As previously described, aglycemia caused loss of WM excitability, measured as failure of the CAP. The pattern of CAP loss with aglycemia was unique, because the CAP was largely unchanged for the first 10 to 15 minutes following glucose deprivation (Fig 1A, B). Once the CAP began to fail, all 3 peaks fell in unison (Fig 1A inset). When glucose was restored after aglycemia, the CAP partially recovered to a stable new level (eg, see Fig 1A). The duration of aglycemia dictated the extent of CAP recovery (Fig 1B). The area under the CAP, which is proportional to the number of excited axons (see Stys et al), recovered to 72.2 ± 6.3%, 48.2 ± 3.7%, or 5.0 ± 1.2% of control CAP area after 30, 60, or 90 minutes of aglycemia, respectively. The extent of CAP recovery following a given period of aglycemia stabilized after about 30 minutes and remained stable during observation periods as long as 4 hours. For quantification and comparison, the area of CAP recovery was determined 60 minutes following the conclusion of glucose deprivation.

**Glycogen Content Affected the Extent of Aglycemic Injury**

It is known that tissue glycogen content affects the latency to CAP failure after removing glucose; more glycogen prolonged and less glycogen shortened latency to
The present study focused on CAP recovery after standard periods of aglycemia and the role of glycogen must be considered. As shown in Figure 1C, the extent of recovery was significantly less if glycogen was first depleted by preincubation for 1 hour in 2mM glucose, rather than the “normal” glucose of 10mM.33 With this important variable in mind, all subsequent experiments were done after 1-hour incubation in 10mM glucose to ensure a consistent level of glycogen.

Aglycemic WM Injury Did Not Depend on Animal Age

The extent of WM injury due to ischemia is dependent on animal age; for a given period of ischemia, tissue from older animals shows significantly greater injury.19 Similar experiments were done to test for age-dependency of hypoglycemic WM injury. The extent of injury after 60 minutes of aglycemia, measured as CAP recovery, was determined in 4 groups of animals ranging in age from 1 month to 24 months. The extent of irreversible injury was similar for all age groups, indicating that age did not affect aglycemia-induced injury extent (see Fig 1D), as is the case with ischemia.19

Aglycemic Injury Was Mediated by NMDA Receptors

The mechanism(s) underlying aglycemic WM injury were investigated. AMPA/kainate-type glutamate receptors participate in ischemic WM injury,15,23,30 and we tested for involvement of these receptors in aglycemic

FIGURE 1: Recovery of white matter (WM) function after aglycemia depended on the duration of aglycemia and tissue glycogen, but not on age. (A) Shown is the effect of a standard 60-minute period of aglycemia on normalized compound action potential (CAP) area for a single mouse optic nerve (MON; 3-month-old animal; see Materials and Methods). Inset shows representative CAPs before (a), during (b), and after (c) aglycemia (CAPs elicited every 0.5 minutes). Aglycemia caused CAP failure that recovered in normal glucose to 45% of original CAP area. (B) Effect of aglycemia duration on functional WM recovery. Averaged CAP areas are shown for 3 durations of aglycemia (30, 60, or 90 minutes; n = 8 for each condition). There is a roughly linear decrease in WM recovery (ie, CAP recovery) with increasing duration of aglycemia. (C) Under control conditions (ie, 10mM bath glucose prior to aglycemia), glycogen was present and latency to CAP failure was 11.56 ± 2.04 min (n = 8). After glycogen depletion (see text), latency to CAP failure was 4.33 ± 2.34 minutes (n = 6; p < 4.8 × 10^-5 compared to latency in glycogen-containing MONs). Recovery of function after 60 minutes of aglycemia, measured by CAP area, was significantly greater in glycogen-containing MONs (44.2 ± 4.9% vs 21.8 ± 4.5%; p = 0.0016; see text). (D) Age does not affect the vulnerability of WM to aglycemic dysfunction or injury. There were no significant differences in CAP recoveries after 60 minutes of aglycemia in animals of different ages (1, 3, 10, or 24 months old). Identical experiments done with 90 minutes of aglycemia gave similar results (data not shown). For clarity, standard error of the mean for CAPs was plotted every 3 minutes, not for every CAP.
injury. The dosage of NBQX that was maximally protective for ischemic WM injury had no significant effect on CAP recovery after 60 minutes of aglycemia (control vs NBQX CAP: 49.2 ± 3.7% vs 46.2 ± 5.8%, p > 0.05), and only weakly improved CAP recovery after 90 minutes of aglycemia (Fig 2A; control vs NBQX CAP recovery: 5.0 ± 1.2% vs 12.2 ± 1.8%, p < 0.01).

Blocking NMDARs does not prevent irreversible loss of WM function following ischemia,19,34 despite reducing structural injury to oligodendrocyte processes and myelin.25,35 The NMDA antagonist MK801 was tested and, surprisingly, this agent powerfully improved CAP recovery after both 60 (see data summary below) and 90 minutes of aglycemia (Fig 2A; control vs NBQX CAP recovery: 6.4 ± 1.3% vs 34.9 ± 4.1%, p < 0.001).

It is possible that the high dose of NBQX used in these experiments, 30 μM, might have weakly, and nonspecifically, blocked NMDARs in our system.36 If so, no additional benefit would be expected if NBQX were added to MK801. We found exactly that result (see Fig 2C). The amount of CAP recovery seen after 90 minutes of aglycemia was greatly improved by MK801, compared to control, but CAP recovery did not improve beyond this level when NBQX was added (see Fig 2C). These results strongly argue that NMDARs were the principal, probably the only, type of glutamate receptor responsible for irreversible CAP loss following aglycemia.

NMDARs are modulated in a highly characteristic manner by several factors, including [Mg2+]o, [glycine]o, and pHo.37 Physiological [Mg2+]o, blocks the NMDAR pore at normal membrane potential. Reducing [Mg2+]o by application of Mg2+-free ACSF would be expected to overcome this block and facilitate pore opening and ion flux upon NMDAR activation. The effect of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5). The worsened outcome with application of Mg2+-free ACSF on aglycemia-induced WM injury was determined (Fig 3A). In the presence of greatly reduced [Mg2+]o, 60 minutes of aglycemia was much more damaging; CAP recovery decreased from 48.05 ± 5.3% to 14.9 ± 4.4% (p < 4.1 × 10^-5).
ischemia + 0 Mg$^{2+}$ recovery = 28.2 ± 5.5%, p = 0.43; data not shown). This result eliminated concern about a nonspecific effect of reducing [Mg$^{2+}$].

The NMDAR is unique in requiring 2 agonists for activation, glutamate and glycine (or d-serine).\textsuperscript{37} We reasoned that glycine availability might limit the extent of NMDAR activation during aglycemia, and tested this possibility by providing glycine (1mM) in ACSF during aglycemia (see Fig 3B). Increasing glycine exogenously during aglycemia led to significantly less recovery (36.51 ± 7.00% vs 48.19 ± 3.70%, p < 0.03). This result supported the idea that NMDARs were involved in aglycemia-induced WM injury and that [glycine] during aglycemia (see Fig 3B). The synchronous initiation of changes in extracellular lactate and pH during aglycemia (36.51 ± 7.0%, n = 8 vs 48.19 ± 1.7%, n = 19; p < 0.03). The presence of glycine also accelerated CAP decline during aglycemia. All experiments were done on 3-month-old animals.

**Extracellular Lactate Fell Rapidly during Aglycemia Accompanied by an Alkaline Shift**

Extracellular lactate was measured directly in the MON using a commercially available "enzyme" electrode specific for lactate (see Materials and Methods and Brown et al\textsuperscript{38}). The size of the electrode necessitated measurement at the pial–glial boundary of the nerve as shown diagrammatically in Figure 4A. In the presence of normal bath glucose (ie, 10mM), [lactate$^{-}$]$_{o}$ was zero in the bath and averaged 0.46 ± 0.02 mM ($n = 6$) when the electrode was pressed against the MON. This value reflected [lactate$^{-}$]$_{o}$ within the optic nerve but will generally be less than the "true" nerve [lactate$^{-}$]$_{o}$ because of an unknown amount of bath dilution (see Brown et al\textsuperscript{38}). The [lactate$^{-}$]$_{o}$ in the nerve was stable over time and did not change when bath glucose was lowered to 5mM or increased to 20mM (data not shown; see Discussion).

During aglycemia, [lactate$^{-}$]$_{o}$ declined rapidly after about 5 minutes and fell to near zero in about 15 minutes (see Fig 4B). pH$_{o}$ within the optic nerve was measured using pH-sensitive microelectrodes placed toward the center of the nerve. As previously reported, pH$_{o}$ was about 0.2 pH units more acid than the bath pH ($\sim$7.4).\textsuperscript{6,39} The fall in [lactate$^{-}$]$_{o}$ during aglycemia was accompanied by a temporally related increase in pH$_{o}$ that ultimately plateaued at about 7.4, near the bath pH (see Fig 4C). The synchronous initiation of changes in [lactate$^{-}$]$_{o}$ and pH$_{o}$ occurred well before the CAP began to fall. When the CAP reached its minimum during aglycemia, an inflection point was often seen in the alkaline shift of pH$_{o}$, suggesting that there were 2 stages to the alkaline shift (see Fig 4C; see Discussion). After switching back to normal bath glucose, [lactate$^{-}$]$_{o}$ and pH$_{o}$ rapidly returned to their original values, although [lactate$^{-}$]$_{o}$ transiently overshot its baseline level.

The proton-binding site on the NMDAR renders it exquisitely sensitive to pH$_{o}$. In other words, protons directly inhibit NMDARs, with a median inhibition concentration value that corresponds to physiological pH.\textsuperscript{37,40} Acidic shifts in pH$_{o}$ block, whereas alkaline shifts facilitate, NMDAR-mediated ion fluxes. Aglycemia caused an alkaline shift in pH$_{o}$ due to the fall in [lactate$^{-}$]$_{o}$, which would enhance NMDAR-mediated ion fluxes and, presumably, injury. This idea was tested by altering pH$_{o}$.
during aglycemia (see Fig 4D; see Materials and Methods). The aglycemia-induced injury was compared in bath solutions identical except for pH (7.45 vs 7.20; see Fig 4D). CAP recovery after 90 minutes of aglycemia was significantly greater in the more acidic bath solution, as predicted if proton-sensitive NMDARs are a primary step in the pathogenesis of aglycemic WM injury. Interestingly, the latencies to the start of CAP decline and to complete CAP loss were both markedly delayed in the acidic solution. We reasoned that if the exogenous acid shift blocked NMDARs, this manipulation would not be additive to the protection afforded by MK801. The results showed that CAP recovery from 90 minutes of aglycemia in the combined presence of the acid shift and MK801 was not significantly different than recovery after exposure to MK801 alone (28.1 ± 6.5% vs 30.8 ± 5.5%, respectively; not significant; data not shown).

**Aglycemic WM Injury Was Dependent on [Ca\(^{2+}\)]\(_o\)**

An important feature of NMDARs is high permeability to Ca\(^{2+}\), and this characteristic can lead to neural injury. If NMDARs are involved in mediating aglycemic WM injury, a high degree of dependence on [Ca\(^{2+}\)]\(_o\) is expected. In experiments on 3-month-old MONs, the extent of CAP recovery after 90 minutes of aglycemia was enormously increased when the insult occurred in the absence of extracellular Ca\(^{2+}\) (from 4.97 ± 1.23% to 86.93 ± 3.6%, p < 0.00001; Fig 5A). It was previously noted in older animals (ie, >6 months of age) that exposure to Ca\(^{2+}\)-free ACSF in conjunction with ischemia caused a delayed CAP deterioration following the insult.\(^{19}\) This paradoxical result, still unexplained, was not seen in older animals exposed to aglycemia in the presence of Ca\(^{2+}\)-free ACSF. In other words, Ca\(^{2+}\)-free...
ACSF afforded dramatic and persistent protection against aglycemic WM injury regardless of age (see Fig 5B). Experimental results from the above experiments are summarized in Figure 6. The results from 90 minutes and 60 minutes of aglycemia are shown in panels A and B, respectively. Note that NBQX did not improve CAP recovery from 60 minutes of aglycemia (see Fig 6B). Although NBQX had a small protective effect against injury due to 90 minutes of aglycemia, this was not additive to the much greater MK801 protection. Taken together, these results indicated that AMPA/kainate receptors were not involved in mediating aglycemic injury, or at most had a minimal effect compared to NMDARs.

Amino Acid Release during Aglycemia

Glutamate is robustly released during ischemia in WM and activates AMPA/kainate receptors that mediate irreversible WM injury. Aglycemic injury, conversely, occurred through activation of NMDARs with little or no involvement of AMPA/kainate receptors (see above). Experiments to detect glutamate release during aglycemia were performed. Using HPLC, glutamate concentration was measured in sequential aliquots of bath solution passing over the optic nerve. Under control conditions, glutamate release monitored in this manner was low and remained stable for at least 3 hours. No apparent change in glutamate release was detected during 40 minutes of aglycemia (see Fig 7A). When aglycemia was switched to oxygen/glucose deprivation (ie, ischemia), however, glutamate release increased almost 10-fold over about 35 minutes, as previously reported. Aspartate can also activate NMDARs and might be involved in hypoglycemic injury of GM areas like the hippocampus. Like glutamate release, no apparent change in aspartate release could be detected during aglycemia, but it was clearly released by a following episode of OGD (see Fig 7B). Insets show these data quantitatively (see Fig 7A, B). Longer periods of aglycemia, up to 90 minutes, also failed to demonstrate any obvious change in glutamate or aspartate release (not shown).

These results did not eliminate the possibility of subtle changes in glutamate or aspartate release during aglycemia. The standard method employed would not be able to detect very small changes in amino acid release into the extracellular space of the optic nerve, because tissue extracellular volume is minute in comparison to bath volume, introducing a large dilution factor. To increase the resolution for detecting small amounts of amino acid release, further experiments were done using 10 MONs rather than 2 (see Materials and Methods). Because the volume of bath perfusion solution remained unchanged, or was slightly less because of displacement by the 8 additional nerves, this strategy magnified the concentration of released amino acid by at least a factor of 5. Using this modification, a decrease in glutamate release was clearly detected during aglycemia (see Fig 7C). It also became clear that aspartate release actually increased significantly during aglycemia (see Fig 7D). These data are shown quantitatively in panels E and F of Figure 7. The concentrations of the NMDAR coagonists, glycine and d-serine, were also measured, and their release was not significantly changed during aglycemia (data not shown).

A further analysis of amino acid changes during aglycemia was carried out by measuring amino acid...
content of MON tissue under control conditions and after 30 minutes of aglycemia (see Fig 7G). This provided a measure of intracellular amino acid content. During aglycemia, intracellular aspartate concentration increased about 4-fold, whereas intracellular glutamate concentration decreased by at least 4-fold. Intracellular glycine concentration did not change significantly during aglycemia. As explained in the discussion, these findings provide a rational explanation for the measured changes in the release of glutamate and aspartate during aglycemia (ie, see Figs 7C–F).

**Discussion**

Hypoglycemia is a common cause of neurologic symptoms.\(^1\)–\(^4\),\(^41\) If severe and prolonged, hypoglycemia causes irreversible neural injury.\(^3\) Most studies on the mechanisms of hypoglycemic brain dysfunction and/or injury have focused on GM. This is unfortunate because WM also suffers from glucose deprivation and damage to this region contributes to clinical deficits,\(^10\),\(^11\) which is not surprising, as WM represents a major portion (about 55%) of human forebrain volume.\(^42\) Moreover, the mechanisms of WM injury are distinctive compared to GM\(^43\) (see below). Finally, recent clinical reports show that WM can be selectively and severely injured by hypoglycemia.\(^10\),\(^11\) Therapy for severe hypoglycemia, therefore, must benefit both WM and GM to be clinically effective.

We studied WM hypoglycemic injury and found that this injury was caused, in part, by excitotoxicity mediated by NMDARs, most likely activated by aspartate released into the extracellular space. This is unexpected, because a related insult, ischemia (ie, OGD), injures WM via AMPA/kainate receptors, and NMDARs are not involved in producing irreversible loss of WM excitability\(^19\),\(^23\) (see below). Our conclusion is supported by the following observations: (1) hypoglycemic injury was significantly blocked by NMDAR antagonists (but minimally by AMPA/kainate receptor antagonists); (2) aspartate release was detected during aglycemia, and aspartate activates NMDARs but not other glutamate receptors; (3) glutamate release fell during aglycemia, precluding activation of AMPA/kainate receptors; (4) conditions known to favor activation of, and ion permeation through, NMDARs (reduced \([\text{Mg}^{2+}]_o\), and increased \([\text{glycine}]_o\) worsened injury; (5) during aglycemia, pH\(_o\) increased (ie, proton concentration fell) due to a fall in \([\text{lactate}]_o\), relieving the “proton block” that reduces ion fluxes through activated NMDARs; and (6) aglycemic injury was greatly mitigated by lowering \([\text{Ca}^{2+}]_o\), as would be expected if injury depended on NMDAR-mediated \(\text{Ca}^{2+}\) influx. These findings have important implications for the clinical management of severe hypoglycemia, because WM may be among the first brain regions affected.\(^11\) They suggest that early treatment with...
FIGURE 7.

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a suitable antagonist of NMDARs may be beneficial. Beyond this, they clarify the importance of extracellular ionic environment as a precondition for NMDAR involvement in neural injury.

**Amino Acid Release during Hypoglycemia**

In WM, one of the most striking differences between ischemia and aglycemia is the pattern of amino acid release induced by the two conditions. During ischemia, glutamate and aspartate are released into the extracellular space after a latency period of about 20 minutes (see Fig 7 A, B). The latency is directly related to the time necessary for astrocyte glycogen to be depleted. 

During aglycemia, in marked contrast, glutamate release into the extracellular space actually decreased (eg, see Fig 7C, E). Given that ischemia and aglycemia both result in marked loss of tissue adenosine triphosphate (ATP), and that this is associated with failure of extracellular glutamate homeostasis powered by ATP-dependent uptake, the absence of increased glutamate release into the extracellular space during aglycemia was unexpected. Decreased [glutamate]o might be specific to WM, because increased [glutamate]o has been detected in GM regions during hypoglycemia.

The likely explanation is the well-known ability of brain tissue, including WM, to utilize substrates other than glucose for energy metabolism (eg, Brown et al). Although glucose is the primary energy substrate transported across the blood–brain barrier, brain cells themselves can use a variety of substrates as substitutes for glucose, including fructose, glutamine, and ketone bodies. Glutamate may also be used as a fuel. Glutamate, glutamine, and ketone bodies produce energy by entering the Krebs cycle, a metabolic sequence with an absolute dependence on the presence of O₂. This necessity of O₂ is the key for understanding why glutamate release is robust in ischemia and absent in aglycemia (actually, the level of extracellular glutamate fell during aglycemia; see Fig 7C, E). During aglycemia, glutamate, and related metabolites are consumed in a desperate effort to maintain a normal level of ATP. We found that intracellular [glutamate] fell to 25% of control level (see Fig 7H). In ischemia, however, the Krebs cycle is paralyzed due to the absence of O₂, and glutamate accumulates, available for export to the extracellular space (see Fig 7A).

The final piece of this metabolic puzzle relates to aspartate, and why it predominates during aglycemia. In the absence of glucose and the consequent glycolytic production of pyruvate, the Krebs cycle intermediate, oxaloacetate, accumulates and lacks its normal condensation partner, acetyl-CoA (derived directly from pyruvate). Elevated intracellular oxaloacetate, in turn, drives aspartate production via the aspartate–glutamate transaminase reaction. Our data indicated that there was roughly a 4-fold increase of intracellular [aspartate] during aglycemia (see Fig 7G). Aspartate, as well as glutamate, is transported by Na⁺-dependent glutamate transporters. During aglycemia, this process can be expected to run in reverse and export aspartate to the extracellular space (see Fig 7D, F). Evidence for this metabolic sequence of events has been noted for more than a quarter century, but all prior studies have been on GM areas of the brain.

**Model of Aglycemia-Mediated Excitotoxic WM Injury**

The observations reported here indicate that aglycemia sets in motion a unique cascade of events producing irreversible CNS WM injury. Before presenting this model in detail, however, an important loose end must be addressed. The critical involvement of NMDARs in aglycemic WM injury begs the question of where these receptors are located. Experimental evidence indicates that oligodendrocytes are probably the main cell type in WM expressing NMDARs. The critical functional subunit of NMDARs, NR1, was expressed in rodent...
FIGURE 8. Yang et al: NMDAR and WM Injury

A Normal

- myelin
- axon
- $pH_{o} \sim 7.2$
- glutamate ($\sim 1 \mu M$)
- oligodendrocyte
- glutamate receptor
  - AMPA/kainate
  - NMDA
- glucose $\rightarrow$ glucose $\rightarrow$ glycogen
- astrocyte

B Ischemia

- myelin
- axon
- lactic acidosis
- [glutamate] rises ($\gg 1 \mu M$)
- AMPA/kainate receptors activated
- NMDA receptors blocked by $H^+$
- ischemia: AMPA/kainate 'excitotoxic' injury

SUMMARY
- [glutamate] $< 1 \mu M$
- glutamate receptors not activated
- NORMAL WM FUNCTION

FIGURE 8.
optic nerves of all ages. A mouse has been created in which NR1 is selectively deleted in oligodendrocytes (F. Kirchhoff, personal communication). Optic nerves from animals lacking oligodendrocyte NR1 showed a 60% reduction in total NR1 content. The residual NR1 expression appeared to be located on astrocytes and/or NG2 cells (F. Kirchhoff, personal communication). Based on these findings, we may conclude that NMDARs are predominately expressed on oligodendrocytes. Moreover, NMDARs on oligodendrocytes in the optic nerve are clearly functional and mediate Ca\(^{2+}\) influx when activated. The exact distribution of NMDARs on oligodendrocytes in older animals remains unexplored, but in young or developing cells of this lineage, NMDARs are expressed primarily on processes and myelin. The extent to which WM astrocytes or NG2 cells, or possibly even axons, express functional NMDARs is simply not known at this time, so it is prudent to remain open to the possibility that such receptors, to the extent they exist, might be involved in hypoglycemic WM injury.

In normal WM, the supply of O\(_2\) and glucose is sufficient to maintain adequate levels of ATP (Fig 8A). The function of WM is safeguarded by the presence of glycogen in astrocytes, which can serve as an emergency

FIGURE 8: Model comparing the mechanisms of hypoglycemic and ischemic white matter (WM) injury. (A) WM under “normal” conditions. In the presence of normal O\(_2\) and glucose, astrocytes have adequate adenosine triphosphate (ATP) for the Na\(^+\) pump to maintain a steep Na\(^+\) gradient that powers glutamate (also aspartate) uptake; consequently, extracellular [glutamate\(^-\)] and [aspartate\(^-\)] are low. The pH\(_o\) is \(\sim 7.2\). (B) WM during ischemia. Under ischemic conditions, energy metabolism in astrocytes persists until glycogen is exhausted (#1). Acidosis accompanies ischemia and protons block ion fluxes in N-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs), preventing them from participating in the injury cascade (#2). The intracellular content of Na\(^+\) ([Na\(^+\)]\(_i\)) increases in the absence of ATP (#3), causing glutamate release into the extracellular space via reverse Na\(^+\)-dependent glutamate transport (probably from astrocytes, as shown; #4). In turn, glutamate activates AMPA/kainate receptors on oligodendrocytes and their processes (#5), including myelin, leading to intracellular ionic derangements and irreversible injury (#6; for details, see Tekkok et al\(^{30}\)). (C) WM during hypoglycemia. With severe hypoglycemia, or aglycemia, energy metabolism persists until glycogen and other substrates, such as glutamate, are exhausted (#1). In the absence of glucose, glutamate is consumed in the Krebs cycle as a short-term substrate and its intracellular concentration falls (#2). The Krebs cycle now produces aspartate (from accumulating oxaloacetate), and the intracellular concentration of aspartate increases 4-fold (#3; see text). As ATP falls, the Na\(^+\) pump fails and [Na\(^+\)]\(_i\) increases, leading to reversal of Na\(^+\)-dependent glutamate/aspartate transport (#4). Because intracellular glutamate is decreased, and aspartate is increased, it is aspartate that is transported into the extracellular space (#4). The [lactate\(_o\)] falls, pH\(_o\) increases, and the proton block of NMDARs is relieved (see text; #5). Aspartate is a high-affinity agonist for NMDARs and activates these, causing toxic ion fluxes, especially Ca\(^{2+}\) influx (#6), and damage to oligodendrocytes and myelin (#7). OGD = oxygen and glucose deprivation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]
fuel demand during intense neural activity when fuel demand might outstrip supply or during short periods of glucose deprivation. Extracellular glutamate is maintained at very low levels that are below the threshold for activation of AMPA/kainate receptors. Even if the high-affinity NMDARs on oligodendrocytes are partially occupied by glutamate under physiological conditions, they would not conduct ion fluxes without cell depolarization and relief of the proton block that exists to some extent at physiological pHo.

Before considering the events put in motion by aglycemia, it is instructive to look closely at the pathophysiology of WM injury due to ischemia, the essential condition of stroke (see Fig 8B). This form of metabolic deprivation forms a natural counterpoint to the more selective metabolic disturbance caused by glucose deprivation. Understandably, it was often suggested that these conditions, which both reduce tissue ATP, might consequently damage cells in a similar fashion (eg, Rothman and Olney). This is certainly not the case in WM, and the points of difference between the injurious steps unleashed by these two pathologies highlight remarkable differences of the utmost clinical importance. It is also germane to note that the quality of information about WM ischemic injury is detailed and robust, enjoying substantial cross-validation by independent research groups.

Ischemia causes energy metabolism to fail in all the component cells of WM, including axons, oligodendrocytes, and astrocytes (Fig 8B, #1-6). A functional level of energy metabolism persists in astrocytes until glycolysis is exhausted (#1). In the absence of O2, lactate levels rise and cause an extracellular acidosis (#2). A consequence of acidosis is proton block of NMDARs. With exhaustion of ATP, Na+ increases (#3), causing glutamate release into the extracellular space via reverse Na+-dependent glutamate transport (probably mediated by astrocytes; #4). In turn, glutamate activates AMPA/kainate receptors on oligodendrocytes and their processes, including myelin (#5), leading to intracellular ionic derangements and irreversible injury (#6). The strong acidosis blocks, or diminishes, ion fluxes in NMDARs (#2), minimizing their participation in the injury cascade, as monitored by the extent of functional recovery. Curiously, the posts ischemic appearance of myelin is improved by NMDAR blockade, whereas axon injury remains unchanged. It has been reported that unique NMDARs with exclusive sensitivity to glycine are expressed on myelin. Although such receptors could conceivably participate in ischemic WM injury, because glycine increases during ischemia (Z. Ye and B. Ransom, unpublished observations), that they are not Ca2+ permeable makes this less likely. Not shown in Figure 8B are the ionic derangements in axons that also participate in ischemic WM injury (eg, Stys et al, LoPachin and Stys), although the contribution of these ionic mechanisms may diminish in older animals.

With severe hypoglycemia (Fig 8C, #1-7), energy metabolism fails as astrocyte glycogen is exhausted (#1). Because the Krebs cycle remains functional in the presence of O2, glutamate can be consumed as an alternative fuel, driving down both intracellular and extracellular glutamate (#2). As discussed above, glutamate also interacts with accumulating oxaloacetate to form much higher concentrations of intracellular aspartate (#3). As ATP falls, the Na+-pump fails, [Na+]i increases, and Na+-dependent glutamate/aspartate uptake reverses, leading to increasing [aspartate]o (#4). Aspartate is an exclusive, and high-affinity, agonist for NMDARs and activates these receptors, which are located primarily on oligodendrocyte processes and myelin. Both intracellular and extracellular [lactate] fall due to lactate consumption in the Krebs cycle after conversion to pyruvate (#5). This is associated with an increase in pHo, causing relief of the proton block of NMDARs and leading to toxic ion fluxes, especially Ca2+, that damage oligodendrocytes and myelin (#6 and #7). The damage may be more focused on distal oligodendrocyte processes and myelin, based on the greater density of NMDARs in these areas.

Clinical Implications

It is instructive to compare the mechanisms of hypoglycemic injury in WM and GM. GM probably comes from glutamate-containing synaptic vesicles, because it is reduced by ablation of glutamatergic synaptic terminals. Because glutamate, but not aspartate, activates both non-NMDA, as well as NMDA, glutamate receptors, it is immediately clear that the absence of extracellular glutamate release in WM during hypoglycemia constitutes a major difference in how GM and WM are injured by this deprivation. Antagonism of NMDARs is essential to minimize WM damage, but blockade of both receptor subtypes appears to be important to protect GM.

In summary, our results, coupled with recent clinical studies showing that hypoglycemic encephalopathy is common in WM and may begin there, suggest that patients presenting in hypoglycemic coma may benefit from treatment with an NMDAR antagonist.

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Potential Conflicts of Interest
Nothing to report.

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