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WHITE MATTER HYPOGLYCEMIC INJURY REVEALED BY MULTIPHOTON MICROSCOPY IN TRANSGENIC MICE

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ABSTRACT

Hypoglycemia is a common occurrence in diabetic patients. We investigated the time course of axon and oligodendrocyte structural injury in white matter exposed to hypoglycemic conditions. Acute coronal brain slices (400µm) including corpus callosum were prepared from transgenic mice with neuron specific expression of YFP controlled by the Thy1 promoter (Thy1-YFP; line 2.2; G Feng et al., Neuron 2000) or from oligodendrocyte-specific expression of GFP (PLP-EGFP; B.S Mallon et al., J. Neuroscience 2002) or dsRed (PLP-dsRed; F Kirchhoff et al) controlled by a proteolipid protein promoter. Perfused slices from Thy1-YFP mice were visualized using a multiphoton imaging system (Zeiss 510 NLO; upright microscope), which permitted high resolution time-lapse fluorescence imaging of intact axons deep in white matter slices (up to 120µm) with minimal photodamage. Transient glucose deprivation (45 min) caused delayed structural disruption of YFP-labeled axons, which appeared as beading, fragmentation, and loss of fluorescence intensity 30-60 min after restoration of glucose levels. Application of the AMPA/kainate antagonist, 30µM NBQX, reduced axonal injury even if started immediately following glucose deprivation. Multiphoton microscopy also allowed visualization of structural changes in oligodendrocytes in slices from PLP-transgenic mice. These results indicate that glucose deprivation causes delayed structural disruption in axons, mediated in part by activation of AMPA/kainate glutamate receptors. Transgenic expression of fluorescent proteins allows direct observation of cell-specific structural changes in living tissue.

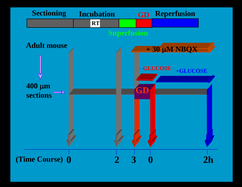

Fig 1. Flow chart of the experimental protocol

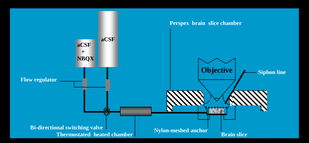

Fig 2. Experimental setup for imaging brain slices

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AXONS

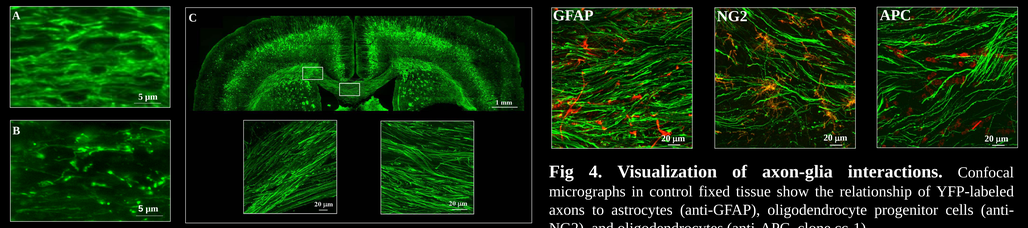

Fig 4. Visualization of axon-glia interactions. Confocal micrographs in control fixed tissue show the relationship of YFP-labeled axons to astrocytes (anti-GFAP), oligodendrocyte progenitor cells (anti-NG2), and oligodendrocytes (anti-APC, clone cc-1).

Fig 3. Thy1-YFP transgenic mouse allows visualization of detailed axonal structure. A: Immunofluoresence micrograph showing SMI-31 labelling of axons in control slice of corpus callosum (c.c). Note linear fibers and bright fluorescence. B: brain slice exposed to 45 min of GD. Note the formation of axonal heads and retraction bulbs and the loss in fluorescence reminiscent of irreversible axonal injury and structural breakdown. C: Montage showing the intense expression and distribution of YFP in cortical layer 5 with axonal projections across c.c and striatum exploiting fluorescence imaging. Below: maximum intensity projections from 16µm fixed sections showing how axonal tracts project along and across the c.c.

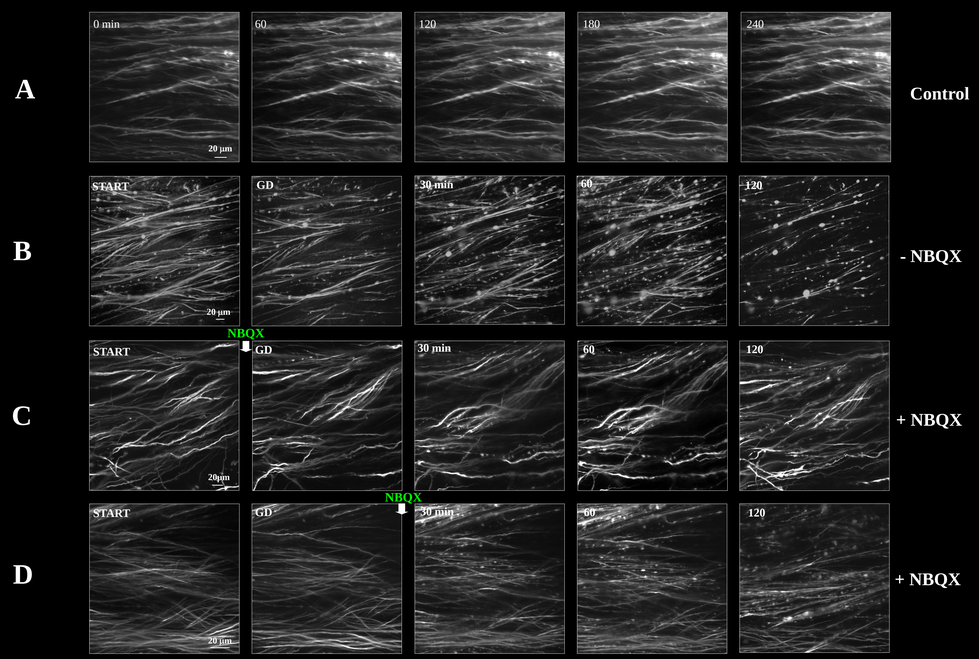

Fig 5. Transient glucose deprivation (GD) causes delayed axonal injury in adult mouse brain slices. AMPA/kainate receptor blockade administered during or after GD substantially preserves axonal structure. Two-photon laser-scanning microscopy permitted high resolution sequential time-lapse imaging deep in white matter with spatial and temporal resolution sufficient to follow morphological changes in axons. Each image is a maximum intensity projection of 20 optical sections spaced 1µm apart, and extending to a depth of 90µm below the slice surface.

Row (a) Brain slice remained structurally intact as long as four hours at ~ 33°C. (b) Slice exposed to 45 min of transient GD showed axonal injury which develops starting 30 min after restoration of glucose. Extensive beading and fragmentation of axons leads to severe axon loss as shown by the progressive loss in fluorescence. (c) Slice exposed to 45 min of GD with NBQX applied 30 min prior, during and after the insult shows well preserved axons. (d) Slice exposed to 45 min of GD with NBQX applied after the insult shows good axonal preservation. Beading and fragmentation of axons is less pronounced and a proportion of axons generally maintain their integrity.

OLIGODENDROCYTES

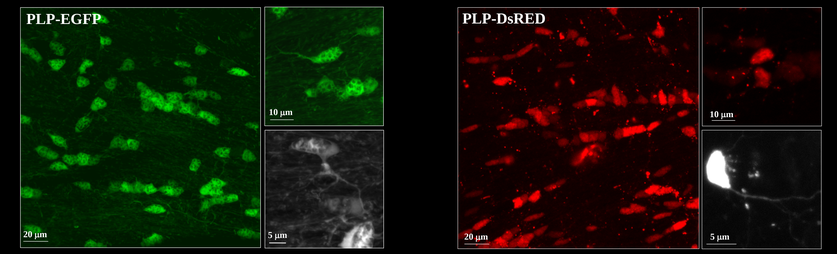

FIG 6. Transgenic mice with oligodendrocyte expression of fluorescent proteins under the proteolipid protein (PLP) promoter. Confocal micrographs show white matter of PLP-eGFP (W. Macklin Lab) and PLP-DsRED (F. Kirchhoff Lab) transgenic mice in control fixed sections (16µm) at low and high magnification. Fluorescence was brighter in mice expressing eGFP than dsRed.

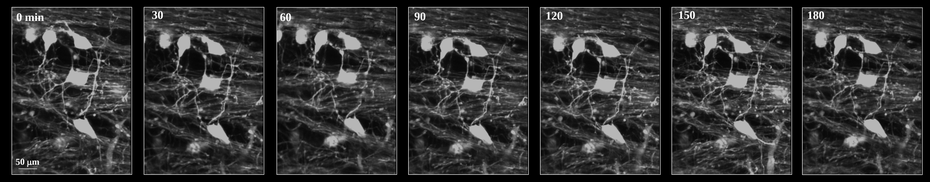

Fig 7. Multi-photon microscopy allowed the sequential imaging of PLP-eGFP oligodendrocytes from live brain slices at high spatial resolution within their native environment. Sequential imaging of acutely isolated brain slice (400 µm) under control conditions shows fine detail of the oligodendrocyte cell bodies and processes in corpus callosum over three hours..

CONCLUSIONS

- **White matter in brain slices from transgenic fluorescent mice remained structurally intact as long as four hours at ~ 33°C, sufficient to assess slowly developing processes such as axonal structural disintegration and oligodendrocyte morphology.**
- **Our acute brain slice model using YFP-H transgenic mice is a reliable model to follow axonal injury following GD, as it allows a detailed sequential assessment of axon morphology, suitable for multiphoton imaging without the reliance on traditional cytoskeleton labelling techniques normally employed in fixed tissue preparations.**
- **In this model, transient glucose deprivation (GD) caused delayed axonal injury, developing 30-120 min after restoration of glucose. NBQX, substantially preserved axon structure even if given after completion of GD.**
- **In the transgenic PLP-EGFP and PLP-dsRED mice, oligodendrocytes could easily be detected in white and gray matter due to their high level of expression. The detailed morphology of PLP-EGFP expressing oligodendrocytes could be visualized at a higher resolution in 16µm-thick brain sections and in living slices (400µm).**

METHODS

Acute brain slice preparation: After deep halothane anesthesia and decapitation, the cranium was opened and the brain rapidly removed and placed in ice-cold and oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid buffer (aCSF) supplemented with 75mM sucrose. aCSF composed of (in mM) 126 NaCl, 3.5 KCl, 1.3 $MgCl_2$, 2.0 $CaCl_2$ 1.2 NaH_2PO4, 25 $NaHCO_3$, and 10.0 glucose, pH 7.4. Sucrose containing physiological saline was composed of 87 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO4, 0.5 mM $CaCl_2$, 3 mM $MgCl_2$, 25 mM glucose, 75 mM sucrose, pH 7.3. After removing the cerebellum and brainstem, the entire brain was mounted on the platform of a Vibratome 1000 vibroslicer (Technical Products, St. Louis, MO), covered in the appropriate buffer (ice-cold and oxygenated) and 400µm thick sections were cut in the coronal plane. Only the slices in which the anatomical structure of the corpus callosum was clearly visualized was included in the experiments. Immediately after sectioning, slices were allowed to recover at room temperature in oxygenated buffer for 2 h in a multi-well Haas-type slice chamber (Scientific Systems Design Inc, Missisauga, Ontario, Canada) at a flow rate of 4.0 ml/min before they were transferred for imaging.

Imaging live brain slice with 2-photon microscopy: A mini submerged chamber (0.5ml) with a coverglass bottom (Warner Instrument Corporation, Hamden, CT) was mounted on an upright Zeiss LSM 5 Pascal laser scanning confocal microscope system. Slices were placed in the submerged chamber and superfused with oxygenated aCSF at a flow rate of 6.0 ml/min. Final temperature control (33 ± 1°C) was maintained using an in-line heater (Warner Instruments) equipped with a feedback thermistor placed in the chamber and raised gradually over 1 h. Perfusion was performed by gravity flow into the chamber and vacuum aspiration within the chamber. A gravity flow regulator in the perfusion line controlled the flow rate. YFP labeled axons were imaged with a 920 nm laser line using a 40X (NA=1.2) water immersion lens through the bottom of the perfusion chamber. For EGFP, imaging was performed at 890nm using a 63X (NA 0.95) water immersion lens. The excitation was generated from a mode-locked Ti:Sapphire laser (Chameleon, Coherent) and images acquired with the microscope operated with external detectors. Twenty z-series optical sections were collected at an incremental z-step of 1µm apart and collapsed to give a maximum intensity projection of each sequential time series. Two-photon laser-scanning microscopy permitted high resolution sequential time-lapse fluorescence imaging deep in white matter slices (up to 90µm) with minimal photodamage. Cutting damage typically extended approximately 40–50µm from the slice surface. Using 2-photon laser scanning microscopy, we were able to image healthy appearing YFP labeled axons restricted to the first 60-90 µm deep were preparation damage was minimal. However, as expected, the fluorescence signal dropped off substantially at greater depths. Glucose deprivation (GD) was initiated by replacing glucose in normal aCSF with 10mM sucrose. For drug application, the oxygenated buffer was replaced with aCSF supplemented with 30 µM NBQX.