# VISUALIZATION OF HYPOXIC OLIGODENDROCYTE INJURY IN PLP-EGFP TRANSGENIC MICE


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## ABSTRACT

White matter injury is an important feature of several acute neurological diseases. To date, none of the immunohistochemical approaches for assessing oligodendrocyte (OL) damage have been entirely satisfactory. We investigated the usefulness of transgenic mice with oligodendrocyte-specific expression of GFP controlled by a proteolipid promoter (Plp-EGFP; BS Mallon et al., J. Neuroscience, 2002), to study the time course of injury during and after 30 min of oxygen-glucose deprivation (OGD). Acute coronal brain slices (400µm) including corpus callosum were transferred to an interface chamber and superfused with aCSF saturated with 95/5% $O_2$/$CO_2$ at 33C. OGD was induced by switching to glucose-free aCSF bubbled with 95/5%$N_2$/$CO_2$. Within 1-2 hours there was widespread OL injury, demonstrated by loss of labeling with OL-specific antibody CC-1 (APC) and gain of pyknotic nuclei. Cytochrome c was released from mitochondria during OGD and diffused thereafter. Confocal visualization of GFP-expressing OLs revealed marked swelling of the nucleus and vacuole formation around the cytoplasm. By 2 hours of reperfusion some of the OLs lost their processes and extensive vacuoles were observed along their entire length. EM confirmed OL injury including swollen mitochondria, clumping of chromatin and cytoplasmic vacuoles. Our results demonstrate close correspondence between Plp-EGFP and EM assessment of OL morphology. The observed damage to OLs matches patterns of white matter injury in other models (Pantoni, 1996; Rosenberg, 1999; Valeriani, 2000). Transgenic expression of other fluorescent proteins controlled by cell-specific promoters allows study of selected cell populations (YFP axons, Plp-dsRed Ols and GFP-GFAP astrocytes) and is a valuable tool to study mechanisms of injury in vivo and in vitro.


## 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 $CaCl_2$, 3.0 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.

**Oxygen-glucose deprivation (OGD):** After the initial superfusion time, aCSF was replaced by glucose-free aCSF (containing 10 mM sucrose to keep the osmolality constant) saturated with a 95% $N_2$/5% $CO_2$ mixture. Slices were sampled right after 30 min of OGD, and after 30, 60 and 120 min reperfusion in glucose containing oxygenated aCSF. These were fixed immediately in 4% paraformaldehyde and 0.025% glutaraldehyde in PBS for 2h at 4ºC. Each slice was halved along its midline for subsequent immunohistochemical and electron microscopy analysis. For immunocytochemistry, brain slices were further dehydrated in 30% sucrose for < 48h at 7ºC before sectioning.

**Immunocytochemistry:** 16µm sections from each condition were blocked and permeabilized in 25% normal goat serum (Sigma) and 0.4% Triton X-100 (Sigma) for 30 min at room temperature. Sections were labelled for oligodendrocytes with monoclonal antibodies CC1 (APC-7; Oncogene Research Products, Cambridge, MA) and cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 and 1:50 respectively and incubated overnight at 4°C. Noncross-reactive donkey anti-mouse CY3 (Jackson ImmunoResearch, West Grove, PA) secondary antibody was used at a dilution of 1:100 in both instances. Nuclear staining was visualized with 0.5 µg/ml Hoechst applied for 1 minute. Sections were mounted with antifade reagent (Molecular probes) and examined by conventional confocal microscopy.

**Electron microscopy:** After an overnight fixation (4°C) in 2%paraformaldehyde, 2% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 each slice (400µm) from each condition was carefully trimmed (2.0 X 2.0 mm) to contain the genu of the corpus callosum (four sections for each condition). Sections were post-fixed for 1 hr in 1% osmium-1% potassium ferricyanide in 0.1 M cacodylate and en bloc stained with 1% uranyl acetate in maleate buffer, pH 6.0, and flat-embedded in epoxy resin (Ted Pella, Inc., Redding, CA). Sagittal ultrathin sections (90-100nm) from the epoxy-embedded tissue blocks were cut with a Leica Ultracut UCT ultramicrotome and subsequently counterstained with uranyl acetate and lead citrate. Sections were viewed at 80 kV on a JEOL 100B TEM with digital image capture.

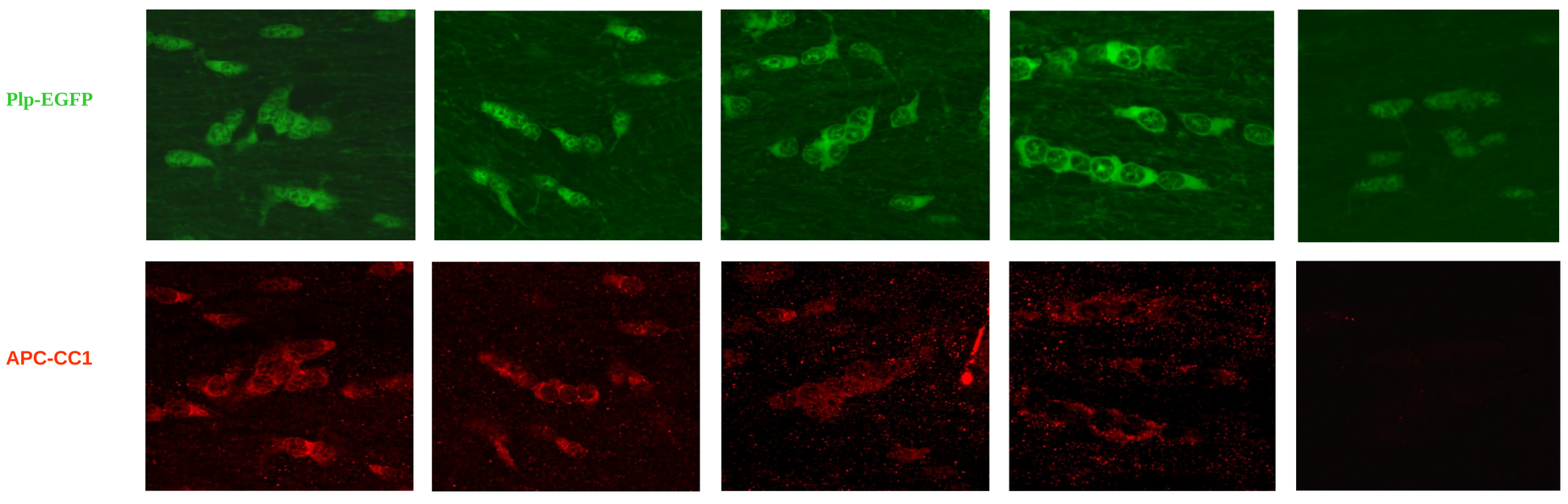


**Fig 1. Transgenic mice with oligodendrocyte expression of specific proteins under the proteolipid protein (PLP) promoter allow visualization of detailed cellular morphology. The sequential loss in CC1 (APC) immunostaining fluorescence intensity preceeds the loss in GFP fluoresence after 30 min of OGD.**

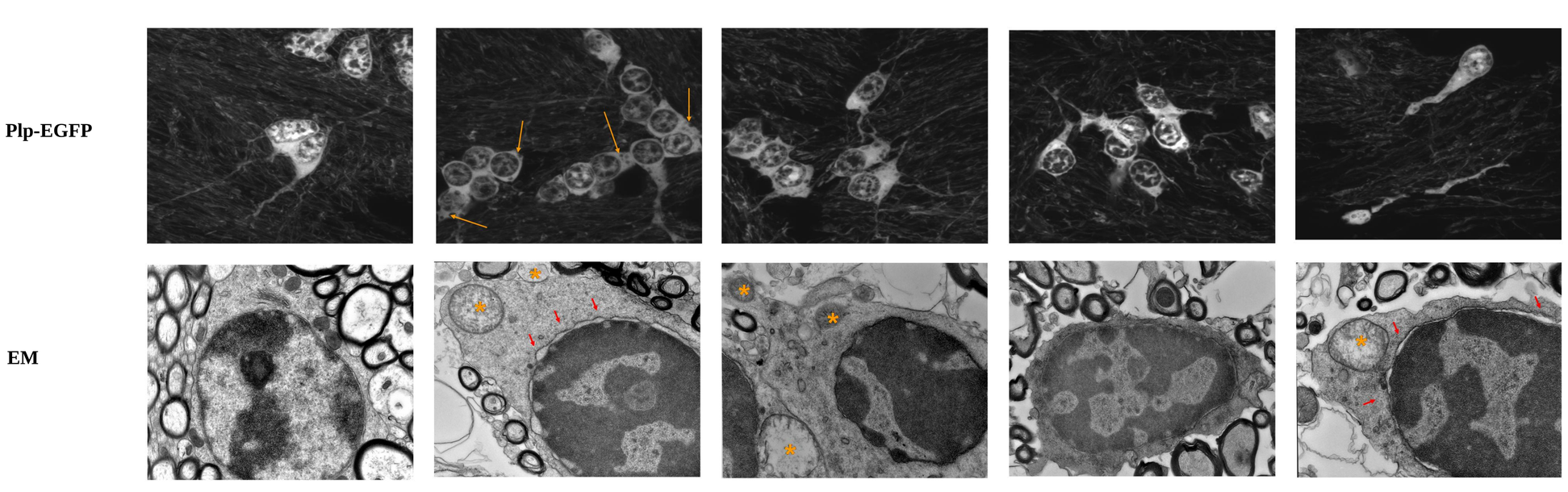


**Fig 2. A close correspondence in structural injury is demonstrated between Plp-EGFP-expressing OLs subject to 30 min of OGD and EM assessment of cellular injury. Structural injury to Plp-EGFP OLs is seen right after 30 min of OGD. There is marked swelling of the nucleus and the appearance of vacuoles in the cytoplasm around the nucleus and processes (arrows). Under EM, this injury corresponds to nuclear swelling, pooling of the chromatin,extensive enlargement of the mitochondria (asterisks) and bleb formation from detachment of the nucleolemma from the nucleus (arrows). By 2h of reperfusion, some OLs show marked detachment of their processes corresponding to severe subcellular injury under EM.**

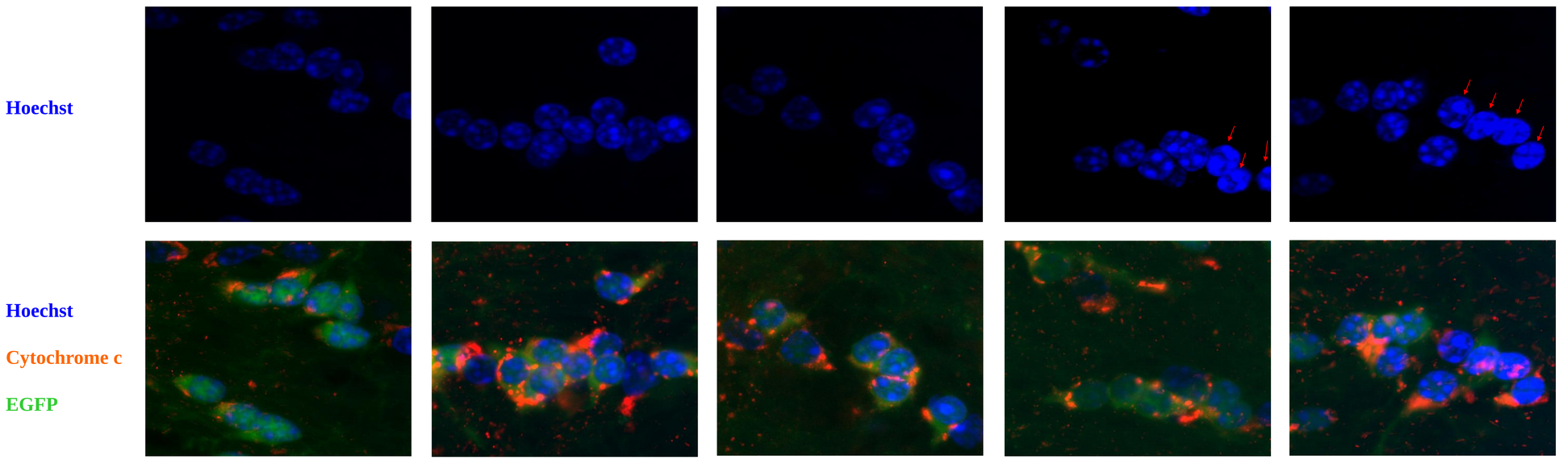


**Fig 3. 30 min of OGD is sufficient to cause release and dispersion of cytochrome c from mitochondria.** Sequential slices (16µm) were immunostained with antibodies to cytochrome c (orange), wheras chromatin was viewed with Hoechst 33258 (blue). Diffuse cytochrome c immunolabeling in OLs but not in control. Release and subsequent diffusion of cytochrome c into the cytosol occurs after OGD. Appearance of condensed nuclei (arrows) is observed after 60 min of reperfusion.

## CONCLUSIONS

- **In the transgenic Plp-EGFP 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).**
- **Our acute brain slice model using Plp-EGFP transgenic mice is a reliable model to follow oligodendrocyte injury following OGD, as it allows a detailed sequential assessment of cellular morphology, (also suitable for multiphoton imaging) without the reliance on traditional labelling techniques normally employed in fixed tissue preparations.**
- **Structural damage to oligodendrocytes is easily manifested as swollen nuclei and the appearance of vacuoles in Plp-EGFP oligodendrocytes right after 30 min of OGD. This injury is confirmed by electron microsopic findings as evidenced by the swollen mitochondria, the loose chromatin in the swollen nucleus and the presence of blebs around the nucleolemma.**
- **The loss in APC immunoreactivity precedes the loss in fluoresence intensity of GFP in Plp-EGFP-labelled oligodendrocytes. Plp-EGFP-labelled oligodendrocytes however allow a more detailed and earlier assessment of structural damage during and after OGD.**
- **OGD causes release of cytochrome c from mitochondria and corresponds to structural damage as evidenced by Plp-EGFP-labelled oligodendrocytes and by electron microscopic examination (Pantoni, 1996; Rosenberg, 1999; Valeriani, 2000). Our results show a close correspondence between injury caused to Plp-EGFP-expressing oligodendrocytes and assessment by EM.**
- **Oligodendrocyte death initiated by 30 min of OGD exhibits some of the primary features of apoptosis, including release of cytochrome c into the cytosol and chromatin condensation.**