PERSPECTIVE

Neurochemical plasticity of Müller cells after retinal injury: overexpression of GAT-3 may potentiate excitotoxicity

The retina is a multilayered tissue that develops following a central-to-peripheral gradient. Its structure derives from multipotent precursors, as shown through clonal analysis of retinal cell lineage. These progenitors generate diverse cell types, controlled by complex influences of intrinsic and extrinsic factors (Hatakeyama and Kageyama, 2004).

Several types of neurons and one main glia cell constitute the vertebrate retina, constructed in a highly organized manner. Müller cell is the predominant glia of the retina and the last cell type to differentiate. Upon damage or under the influence of growth factor stimulation, they proliferate, de-differentiate and become a source of new neurons for retinal regeneration in fish and, to a much less extent, postnatal chicks. Evidence indicates that some Müller glial cells in mammals also have the potential to proliferate in response to N-methyl D-aspartate (NMDA) treatment and produce bipolar cells and rod photoreceptors (reviewed in Goldman, 2014). Three cellular layers make the final organization of the retina, with each of the six types of neurons and the Müller glia. Selective markers in the adult vertebrate retina (mice for all, except GAT-3 for chick) can identify these elements as shown in **Figure 1**. Opsin and rhodopsin label **photoreceptors**, calbindin identifies **horizontal cells**, CHX10 (C. elegans ceh-10 homeodomain-containing homolog) identifies **bipolar cells**, GAT-3 transporter labels selective GABAergic **amacrine cells, Beta$_{III}$** tubulin (tuj-1) recognizes **retinal ganglion cells (RGCs)** and glutamine synthetase (GS) labels **Müller glia**.

Anti-glial fibrilary acidic protein (GFAP) is used to identify reactive **glial cells** that involve the axons of RGCs that make the optic nerve. Retinal diseases affect millions of patients worldwide affecting photoreceptors, as in age-associated macular degeneration (AMD) and retinitis pigmentosa, or RGCs, as glaucoma or diabetic retinopathy. Several laboratories are looking for the possibility of retinal regeneration based on cellular and or molecular strategies.

Normally, GFAP or nestin (a progenitor marker) do not label Müller cells (**Figure 1**) in the undamaged retina, as opposed to GS, a marker that shows Müller glia processes extend across all retinal layers going from the inner to the outer limiting membranes (**Figure 1**).

Traumatic lesion of the retina induces Müller cell proliferation due to inflammatory processes. This condition is mimicked artificially by neurotoxins like NMDA or kainate injected into the eye of postnatal chick (Fischer and Reh, 2001). This procedure makes Müller glia to acquire neurogenic potential in response to injury providing a source of neural stem cells in this tissue. Several pathways seem to be involved with Müller glial proliferation and dedifferentiation such as Notch, initially discovered in zebrafish (reviewed in Goldman, 2014). Notch plays a central role in the conservation of stemness throughout retinal development. Notch-signaling components are expressed at low levels in healthy Müller glia in the postnatal retina, but upon stimulation with basic fibroblast growth factor (FGF2) and insulin, Müller glia proliferate and dedifferentiate. Tumor necrosis factor alpha (TNF-α) together with repression of Notch induce Müller glia to proliferate in the adult zebrafish retina, generating neuronal progenitor cells (reviewed in Goldman, 2014). Wnt/β-catenin also induces proliferation of Müller glia-derived progenitors and regeneration after damage, or during degeneration in the adult rodent retina (reviewed in Goldman, 2014). Finally, sonic hedgehog (Shh) has been shown to stimulate Müller glial proliferation through its receptor. Shh-treated Müller glial dedifferentiate through expression of progenitor-specific markers, leading to the fate of rod photoreceptor. Together, these results provide evidence that Müller glia operate on diverse signaling mechanisms (for a complete list of factors acting on Müller cells, see Goldman, 2014) to reprogram and generate progenitors in zebrafish and perhaps give a clue as potential stem cells in mammalian retina.

Examples depicting the phenotypic plasticity of Müller cells after injury have been described in different vertebrate models. For instance, spatiotemporal distribution of retinal cells induced by lesion is shown in the adult zebrafish (Yurco and Cameron, 2005). They show double labeling immunohistochemistry using proliferation (anti-BrdU or anti-PCNA) and Müller glial markers (carbonic anhydrase, or GS). Müller cell proliferation is also shown in postnatal chick (Fischer and Reh, 2001). Finally, in mammals, few Müller glial cells injected in the adult rat retina to stimulate proliferation produce bipolar cells and rod photoreceptors (reviewed in Goldman, 2014).

Müller glia obtained from rat retina can generate clonal spheres capable of differentiating into functional neurons (Das et al., 2006); In addition, retinal neurospheres from postnatal mice have the potential to generate neurons and Müller glia as identified by calcium imaging protocols (De Melo Reis et al., 2011). The possibility to obtain different types of retinal cells from precursors raise the possibility of developing cell transplants methodologies to restore proper visual function lost in retina degeneration.

The elimination of neurons from mixed retinal neuron-glia cultures makes Müller cells to express several markers found in neuronal cells. Among these, glutamate decarboxylase (GAD), TH, pituitary adenylate cyclase-activating peptide (PACAP) receptors (Kubrusly et al., 2005) and Nurr1, a transcriptional factor associated with dopaminergic phenotype were described. Dopamine D1 receptors are also functional as they generate cyclic AMP. Consequently, purified cultures of Müller cells develop the full complement of functional dopaminergic phenotype, including the release of dopamine. This seems to be due to a default pathway for Müller cells under this condition. This dopaminergic default occurs in Müller cells obtained from avian, mouse and monkey retina (Stutz et al., 2014). Dopaminergic Müller cells

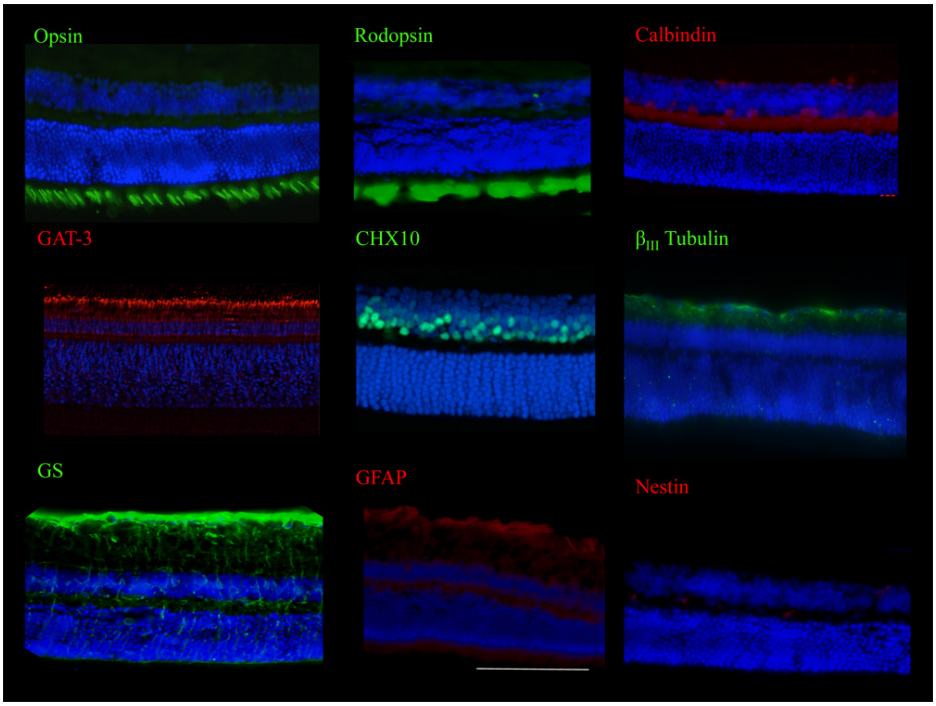

Figure 1 Immunohistochemistry of selective markers of the adult mouse retina.
All animal procedures were approved by the Animal Care and Use Committee of the Biophysics Institute, UFRJ (CEUA permit number IBCCF-126). Efforts were made to minimize animal suffering. C57/BL6 mice were obtained from our department's animal facility. Retina fixation was essentially prepared as described in a study by Schitine et al., (2015). **Opsin** and **rhodopsin** label photoreceptors (cones and rods), **calbindin** identify horizontal cells, **CHX 10** is used to identify bipolar cells, **GABA transporter type 3 (GAT-3)** is used to label GABAergic amacrine cells, **beta$_{III}$ tubulin** (tuj-1) recognizes retinal ganglion cells (RGCs) and **glutamine synthetase (GS)** labels Müller glia. **Glial fibrilary acidic protein (GFAP)** recognizes reactive astrocytes that are present around the axons of the RGCs that make the optic nerve. **Nestin** (a progenitor marker), do not label Müller cells in a healthy retina, as opposed to GS, a marker that shows how Müller glia cross the entire length of the retina extending from the inner to the outer limiting membranes. Scale bar: 100 μm; Primary antibodies used in this study were: Rabbit Policlonal antibody against **S-opsin** (1:1,000; Chemicon; Temecula, CA, USA); mouse monoclonal anti-**Tuj-1** (1:500; Covance; Princeton, NJ, USA); mouse monoclonal anti-**nestin** (1:500; Chemicon), rabbit polyclonal anti-GS (1:800; Abcam; Cambridge, UK), rabbit polyclonal anti-**GFAP** (1:300; Abcam); rabbit polyclonal anti-**Rhodopsin** (1:1,000; Abcam), anti-chx10 (1:2,000; Exalpha Biologicals, Inc.; Shirley, MA, USA); mouse monoclonal anti-**calbindin** (1:300); rabbit polyclonal anti-**GAT-3** (1:500; AB1574, Millipore Billerica, MA, USA). Secondary antibodies were Donkey IgG anti-mouse or anti-Rabbit Alexa fluor 488 conjugated (Molecular Probes) or IgG anti-mouse ou anti-rabbit Alexa fluor 555 conjugated (Molecular Probes), both diluted 1:400. Control retina sections were incubated with PBS in the absence of primary antibody, and no immunoreactivity was detected. For nuclear staining, retinal sections were incubated with DAPI (1:10,000).

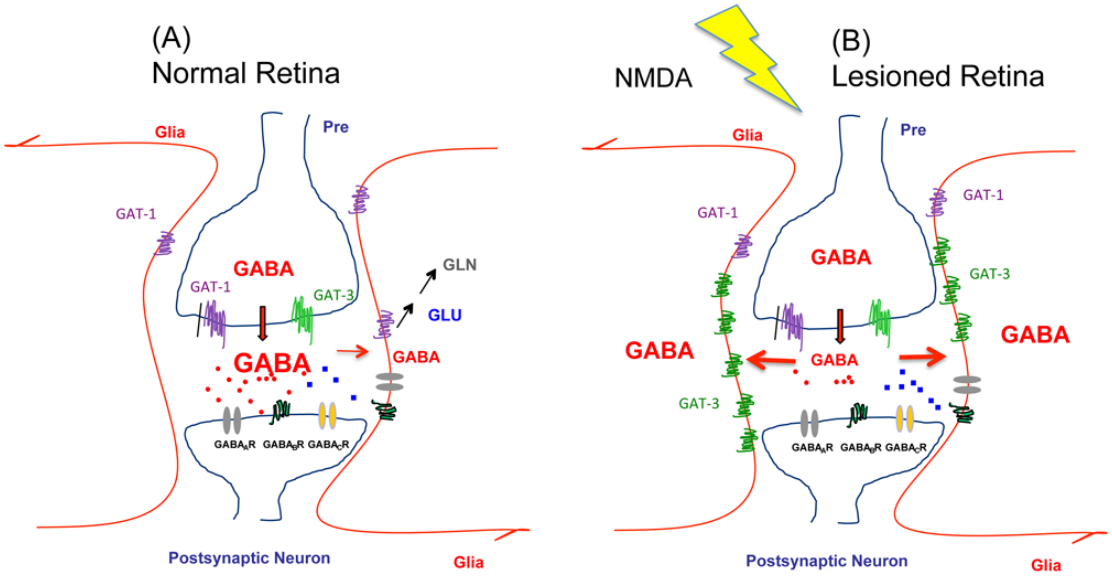

Figure 2 Schematic illustration showing γ-aminobutyric acid (GABA) signaling in avian retina.
(A) In a normal retina, under functional neuron-glia signaling, GABA transporter type 3 (GAT-3) (green) is mainly expressed in amacrine cells in both plexiform and nuclear layers, but is not expressed in Müller glial cells (Schitine et al., 2015). (B) In a lesioned retina induced by intravitreous N-methyl D-aspartate (NMDA) injection, GAT-3 expression pattern shifts from neuron to Müller cells. Therefore, GAT-3 activity decreases GABA levels reducing the inhibitory tonus favoring toxicity.

transplanted into the striatum of hemi-parkinsonian mice fully recover motor behavioral deficits (Stutz et al., 2014). Therefore, it is an attractive possibility to suggest that these dopaminergic Müller cells could be of potential use in cellular therapies for dopaminergic dysfunction. The fact that the dopaminergic default does not require hard manipulation for cells to express the dopaminergic phenotype makes it less likely to cause hazardous influence on healthy tissues.

Müller cells are actively involved in the synaptic control of retinal neurons through the release of transmitters and trophic factors (de Melo Reis et al., 2008). These cells interact with most of the retinal neurons, ranging from RGC to photoreceptors. However, the majority of retinal synapses are glutamatergic and GABAergic in close association with glial cells. In this sense, recent data show that GAT-3, a GABA transporter found in purified Müller glia, is functionally regulated by glutamate. This response involves ionotropic glutamatergic receptors. In addition to GAT-3, GAT-1 is expressed in purified glial cells. However, only GAT-3 seems to be functional (De Sampaio Schitine et al., 2007). Glutamate decreases the levels of GAT-3 transporter in the plasma membrane of Müller cells as well as its mRNA.

In the avian retina, GAT-3 is primarily expressed in the inner plexiform layer (IPL) and in some cell bodies in the INL, where most of the amacrine cells are located (**Figure 1**; Schitine et al., 2015). Müller glia also have their soma in the INL. Retinal lesion induced by NMDA injections provokes a large increase in GAT-3 immunoreactivity in Müller fibers (**Figure 2**), followed by damage to RGCs, and an increase in GFAP expression. Reactive gliosis is a hallmark in several neurologic diseases but not so well understood, and in the retina, it has been associated with several degenerative conditions such as hepatic retinopathy, macular edema, and retinitis pigmentosa.

Evidence from Ortinski et al. (2010) shows that reactive gliosis artificially induced in hippocampal circuits leads to decreased expression of GS, implying a reduction in the glutamate production from glutamine. Therefore, a rapid decrease of GABA content in gabaergic synapses leads to a decreased inhibitory tonus on synaptic transmission in mouse CA1 pyramidal neurons. This seems to favor excitotoxicity. Our recent data suggest that *in vivo* lesions of the retina may be potentiated by decreased inhibitory tonus, due to increased GABA uptake by Müller cells overexpressing GAT-3 (**Figure 2**). Further investigations are necessary to reveal the molecular mechanisms involved in glutamate-dependent GAT-3 plasma membrane level reduction. Interestingly however, our observations open the possibility of using GABA transport inhibitors to prevent RGCs degeneration eventually caused by reactive gliosis that follow retina degeneration.

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