Novel Galectin-3 interactions involved in oligodendroglial differentiation make inroads into therapeutic strategies for demyelinating diseases

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Galectins (Gals) constitute a 15-member class of β-galactoside-binding lectins which recognize *N*-acetyllactosamine. Despite lacking specific receptors, Gals form multivalent complexes with cell surface glycoconjugates containing suitable oligosaccharides and thus trigger intracellular signals to regulate cell survival and differentiation. Gals are classified into three groups on the basis of their structural architecture: proto, chimera and tandem types, with Gal-3 being the only representative of the chimeric type. Regarding intracellular localization, Gal-3 is found in both cell cytoplasm and nucleus. Extracellular Gal-3 can also be endocyted and, together with intracellular Gal-3, modulate diverse functions binding to intracellular molecules. While lacking a secretion signal peptide, Gal-3 is still secreted into the extracellular space by non-classical pathways potentially involving exosomes. Once secreted, Gal-3 binds to poly-N-acetyllactosamine in the extracellular matrix and membrane receptors, laying bridges to promote or inhibit intracellular events (Thomas and Pasquini, 2018).

Myelination is a physiological process by which lipid- and protein-rich myelin wraps axons and provides them with metabolic and trophic support. Having attained maturity through a succession of morphological and molecular changes, oligodendrocytes (OLG) are the cells in charge of central nervous system (CNS) myelination. Starting from highly proliferative platelet-derived growth factor receptor α (PDGFRα)- and neural/glial antigen 2-positive bipolar cells, oligodendroglial progenitors (OPC) later become pre-OLG, more ramified cells expressing CNPase, Olig 1 and O4, to finally develop into mature OLG (m-OLG) with myelin basic protein (MBP), adenomatous polyposis coli and myelin proteolipid protein expression and the ability to form myelin membranes (Franklin and Ffrench-Constant, 2017). A two-stage model has been proposed for actin dynamics in OLG: at an early stage, pro-polymerizing actin cytoskeleton dynamics foster OPC branching up to full OLG maturity; at a later stage, the actin cytoskeleton shifts to depolymerizing dynamics, which sparks myelination. These stages are partly regulated by the relative levels of MBP and actin disassembly proteins, e.g. colfilin-1 and gelsolin, commonly sequestered and inactivated by phosphatidylinositol 4,5-bisphosphate in the plasma membrane. MBP then competes for phosphatidylinositol 4,5-bisphosphate binding with gelsolin and cofilin-1 and displaces them to trigger the disassembly of actin filaments in m-OLG (Zuchero et al., 2018).

In 2011, our group pioneered the study of direct Gal-3 influence on OLG, showing that extracellular microglia-secreted Gal-3 promotes OLG differentiation, as wild type, but not LGALS3$^{-/-}$-microglia-conditioned media, succeeded in inducing OLG maturation. In addition, Gal-3 expression appears to preserve myelin integrity and function, as evidenced by myelin ultrastructural and behavioral studies of LGALS3$^{-/-}$ mice. Glycosylation signature analysis has shown that OPC possess a permissive glycophenotype expressing the necessary carbohydrates for Gal-3 binding (Pasquini et al., 2011). More recently, our group showed accelerated differentiation in recombinant Gal-3 (rGal-3)-treated OLG. In this study, rGal-3 administered to purified OPC once every 2 days accelerated differentiation, as evidenced by an early increase in m-OLG markers and a decrease in immature OLG ones. These results coincided with accelerated F-actin dynamics, as revealed by an earlier polymerization peak and subsequent depolymerization, and were accompanied by an increase in the activation of Akt and the levels of β-catenin, MBP and gelsolin, as well as a decrease in the activation of Erk1/2 (Thomas and Pasquini, 2019). Even though how and when exactly the Akt/mTORC and Erk1/2 pathways participate in OLG differentiation and myelination remain controversial, a recent report has shown that PI3K/Akt, but not Mek/ErK1/2, plays a key role in promoting OLG differentiation. However, these two pathways work together converging at the level of mTORC1 to produce a mature and functional myelin (Thomas and Pasquini, 2020). In addition, other authors have postulated that Erk 1/2 inhibition promotes OLG generation and recovery in demyelinating processes (Thomas and Pasquini, 2020).

The timing and mechanisms underlying the pro-differentiating effects of Gal-3 were addressed through a series of treatment protocols using a single pulse of rGal-3 applied to different OLG maturation stages, i.e., OPC, pre-OLG and m-OLG. In these studies, rGal-3 was found to increase MBP expression, pAkt, β-catenin and F-actin, and to accelerate actin dynamics at both OPC and pre-OLG stages. Our results also indicate that mTORC1 is activated by rGal-3 treatment, which contributes to MBP expression and OLG maturation. However, single rGal-3 treatment at m-OLG stage failed to increase MBP expression, F-actin or β-catenin levels, and to activate Akt. Furthermore, all treatments induced an increase in gelsolin concomitantly with a decrease in pErk 1/2. These effects may be linked to increased MBP expression, as higher gelsolin levels positively correlate with OLG differentiation. Altogether, these results hint not only at a time window for rGal-3 action spanning OPC and pre-OLG stages, but also at interactions occurring throughout OLG maturation, which reinforces the key role of glycolipids and glycoproteins present at the time of treatment (Thomas and Pasquini, 2020).

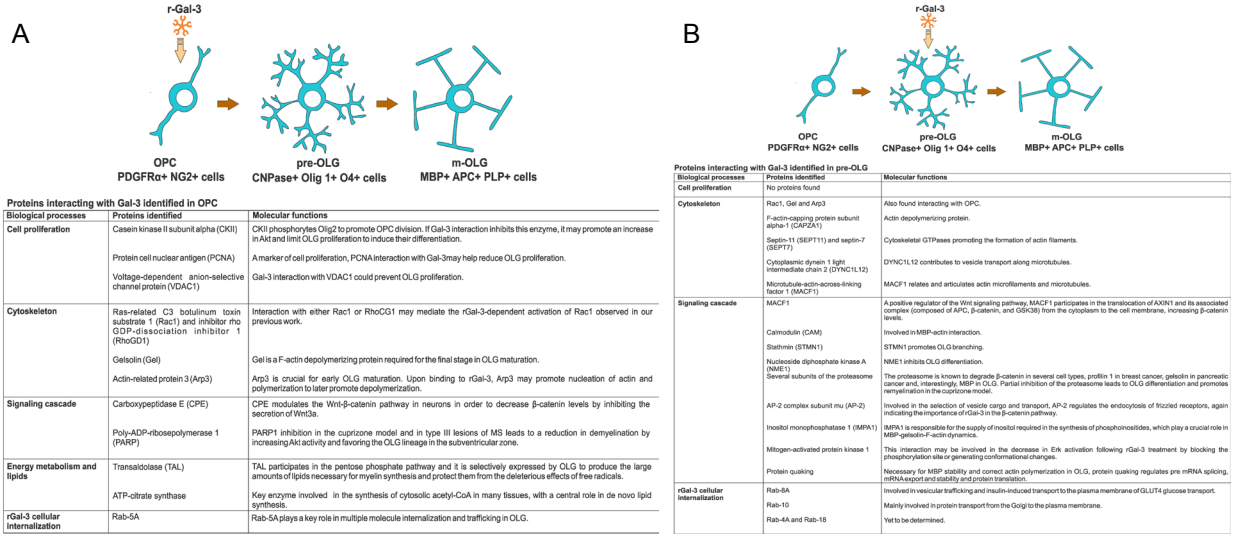
Finally, direct rGal-3-molecule interactions were evaluated in OPC and pre-OLG by co-immunoprecipitation and subsequent mass spectrometry analysis. The main proteins found to interact with rGal-3 classified on the basis of the biological processes involved and their possible function in OPC are summarized in **Figure 1A** and **B** following the information previously described in Thomas and Pasquini (2020). Interestingly, the proteins identified at OPC stage are associated to cell proliferation, cytoskeleton, signaling cascades, energy metabolism and lipids and, probably, Gal-3 cellular internalization. At variance, proteins found to interact with rGal-3 at pre-OLG stage include those related to cytoskeleton, signaling cascades and rGal-3 cellular internalization, but none involved in cell proliferation. The characteristics of these proteins and their association to specific OLG stages support the notion that, upon internalization, extracellular rGal-3 is distributed into different subcellular compartments to interact with target molecules and thus mediate changes in the cytoskeleton, proliferation, lipid synthesis, and signaling pathways necessary to attain OLG differentiation.

Oligodendroglial injury leads to demyelination, the pathological process by which myelin is lost from around axons (Franklin and Ffrench-Constant, 2017). Among demyelinating diseases, multiple sclerosis (MS) is one of the most common neurodegenerative CNS disorders, affecting 2.5 million people worldwide. Remyelination, the regenerative response consisting in the formation of new myelin sheaths, is essential for axonal survival and restoration of saltatory conduction, whereas its failure is a major cause of the neurological deficits in MS. Given that unsuccessful remyelination may result from inefficient removal of myelin debris by microglia, unveiling the mechanisms controlling phagocytosis may prove an effective approach to reversing disability and curbing disease progression.

The endogenous and exogenous roles of Gals in glial cells upon demyelination/remyelination have been recently documented in a thorough review by Jong et al. (2018). Serum from patients with secondary progressive MS has recently shown the presence of autoantibodies against Gal-3, probably responsible for blood-brain barrier progressive damage given their ability to bind to membrane Gal-3 in human brain microvascular endothelial cells (Thomas and Pasquini, 2018). Further evidence regarding the effect of cerebrospinal fluid from patients with primary progressive MS or relapsing remitting MS on OPC morphology and transcriptome has been provided. OPC treated with primary progressive MS cerebrospinal fluid exhibit more ramified morphology than those treated with control or relapsing remitting MS cerebrospinal fluid and show a pro-differentiating transcriptome, i.e. the downregulation of PDGFRα and LINGO1 genes and the upregulation of MAG gene, although this transcriptome differs from that found in normal OPC differentiation. Of note, this report shows LGALS3 gene upregulation only in OPC treated with primary progressive MS cerebrospinal fluid and postulates a connection between Gal-3 upregulation and OPC branching. Worth pointing out, Gal-3 upregulation has also been detected in post-mortem human brain tissues with primary progressive MS (Thomas and Pasquini, 2018). Likewise, when administered upon EAE immunization, FDA-approved drug for MS glatiramer acetate (copolymer 1) decreases Gal-3 expression in macrophages in the lesion site (Jong et al., 2018). Altogether, these findings hint at Gal-3 as a positive target for OLG differentiation in human MS tissue and point at the downregulation of ICAM-1 in brain microvascular endothelial cells as a means to protect the blood-brain barrier. However, the harmful effects of anti-Gal-3 auto-antibodies on the blood-brain barrier should be counteracted through neutralizing therapy.

Although MS experimental demyelination models fail to replicate the full complexity and heterogeneity of MS features, they have rendered interesting results and have allowed the development of various treatments. EAE applied on LGALS3$^{-/-}$ mice rendered a reduction in CNS macrophage infiltration and disease severity, indicating a key role for Gal-3 in the promotion of inflammation by leukocyte recruitment (Thomas and Pasquini, 2018). At variance, further studies in this model revealed a neuroprotective role of Gal-3 through cell debris removal, axon regeneration and remyelination (Thomas and Pasquini, 2018). An increase in Gal-3 expression has been observed in active human MS lesions, periventricular regions in human MS and after virus-induced MS models like Theiler's murine encephalomyelitis virus, in which the loss of Gal-3 restores subventricular zone proliferation through a reduction in the number of immune cells (Thomas and Pasquini, 2018).

Our results have demonstrated that, during remyelination, microglia-expressed Gal-3 favors an M2 phenotype, hence fostering myelin debris phagocytosis through TREM-2b phagocytic receptor and OLG differentiation (Hoyos et al., 2014). Furthermore, recent work has identified Gal-3 as a novel endogenous TREM2 ligand (Boza-Serrano et al., 2019). Also recently, Gal-3 has been shown to activate phagocytosis by targeting the cytoskeleton



Proteins interacting with Gal-3 identified in OPC

Biological processes	Proteins identified	Molecular functions
Cell proliferation	Casein kinase II subunit alpha (CKII)	CKII phosphorylates Olig2 to promote OPC division. If Gal-3 interaction inhibits this enzyme, it may promote an increase in Akt and limit OLG proliferation to induce their differentiation.
	Protein cell nuclear antigen (PCNA)	A marker of cell proliferation, PCNA interaction with Gal-3may help reduce OLG proliferation.
	Voltage-dependent anion-selective channel protein (VDAC1)	Gal-3 interaction with VDAC1 could prevent OLG proliferation.
Cytoskeleton	Ras-related C3 botulinum toxin substrate 1 (Rac1) and inhibitor rho GDP-dissociation inhibitor 1 (RhoGD1)	Interaction with either Rac1 or RhoCG1 may mediate the rGal-3-dependent activation of Rac1 observed in our previous work.
	Gelsolin (Gel)	Gel is a F-actin depolymerizing protein required for the final stage in OLG maturation.
	Actin-related protein 3 (Arp3)	Arp3 is crucial for early OLG maturation. Upon binding to rGal-3, Arp3 may promote nucleation of actin and polymerization to later promote depolymerization.
Signaling cascade	Carboxypeptidase E (CPE)	CPE modulates the Wnt-β-catenin pathway in neurons in order to decrease β-catenin levels by inhibiting the secretion of Wnt3a.
	Poly-ADP-ribosepolymerase 1 (PARP)	PARP1 inhibition in the cuprizone model and in type III lesions of MS leads to a reduction in demyelination by increasing Akt activity and favoring the OLG lineage in the subventricular zone.
Energy metabolism and lipids	Transaldolase (TAL)	TAL participates in the pentose phosphate pathway and it is selectively expressed by OLG to produce the large amounts of lipids necessary for myelin synthesis and protect them from the deleterious effects of free radicals.
	ATP-citrate synthase	Key enzyme involved in the synthesis of cytosolic acetyl-CoA in many tissues, with a central role in de novo lipid synthesis.
rGal-3 cellular internalization	Rab-5A	Rab-5A plays a key role in multiple molecule internalization and trafficking in OLG.

Proteins interacting with Gal-3 identified in pre-OLG

Biological processes	Proteins identified	Molecular functions
Cell proliferation	No proteins found	
Cytoskeleton	Rac1, Gel and Arp3	Also found interacting with OPC.
	F-actin-capping protein subunit alpha-1 (CAPZA1)	Actin depolymerizing protein.
	Septin-11 (SEPT11) and septin-7 (SEPT7)	Cytoskeletal GTPases promoting the formation of actin filaments.
	Cytoplasmic dynein 1 light intermediate chain 2 (DYNC1LI2)	DYNC1LI2 contributes to vesicle transport along microtubules.
	Microtubule-actin-across-linking factor 1 (MACF1)	MACF1 relates and articulates actin microfilaments and microtubules.
Signaling cascade	MACF1	A positive regulator of the Wnt signaling pathway, MACF1 participates in the translocation of AXIN1 and its associated complex (composed of APC, β-catenin, and GSK3B) from the cytoplasm to the cell membrane, increasing β-catenin levels.
	Calmodulin (CAM)	Involved in MBP-actin interaction.
	Stathmin (STMN1)	STMN1 promotes OLG branching.
	Nucleoside diphosphate kinase A (NME1)	NME1 inhibits OLG differentiation.
	Several subunits of the proteasome	The proteasome is known to degrade β-catenin in several cell types, profilin 1 in breast cancer, gelsolin in pancreatic cancer and, interestingly, MBP in OLG. Partial inhibition of the proteasome leads to OLG differentiation and promotes remyelination in the cuprizone model.
	AP-2 complex subunit mu (AP-2)	Involved in the selection of vesicle cargo and transport, AP-2 regulates the endocytosis of frizzled receptors, again indicating the importance of rGal-3 in the β-catenin pathway.
	Inositol monophosphatase 1 (IMPA1)	IMPA1 is responsible for the supply of inositol required in the synthesis of phosphoinositides, which play a crucial role in MBP-gelsolin-F-actin dynamics.
	Mitogen-activated protein kinase 1	This interaction may be involved in the decrease in Erk activation following rGal-3 treatment by blocking the phosphorylation site or generating conformational changes.
	Protein quaking	Necessary for MBP stability and correct actin polymerization in OLG, protein quaking regulates pre mRNA splicing, mRNA export and stability and protein translation.
rGal-3 cellular internalization	Rab-8A	Involved in vesicular trafficking and insulin-induced transport to the plasma membrane of GLUT4 glucose transport.
	Rab-10	Mainly involved in protein transport from the Golgi to the plasma membrane.
	Rab-4A and Rab-18	Yet to be determined.

Figure 1 | **Proteins interacting with rGal-3.**
(A) The main proteins found to interact with rGal-3, obtained through co-immunoprecipitation and mass spectrometry analysis of OPC lysates by Thomas and Pasquini (2019). These proteins are classified according to biological processes and possible molecular functions in OLG, following the information previously reported in Thomas and Pasquini (2019). (B) The main proteins found to interact with rGal-3, obtained through co-immunoprecipitation and mass spectrometry analysis of pre-OLG lysates by Thomas and Pasquini (2019). These proteins are classified according to biological processes and possible molecular functions in OLG, following the information previously described in Thomas and Pasquini, 2019. APC: Adenomatous polyposis coli; CNPase: 2′,3′-cyclic-nucleotide 3′-Phosphodiesterase; MBP: myelin basic protein; m-OLG: mature OLG; mTOR: mammalian target of rapamycin complex; NG2: neural/glial antigen 2; OLG: oligodendrocyte; Olig: oligodendrocyte transcription factor; OPC: oligodendroglialprogenitor; PDGFRα: platelet-derived growth factor receptor α; PLP: myelin proteolipid protein; rGal-3: recombinant Galectin 3; TREM-2: triggering receptor expressed on myeloid cells-2.

twice: first, by advancing cofilin activation, enabling filopodia/lamellipodia to extend/engulf myelin debris; and second, by advancing actin/myosin-based contraction through K-Ras. GTP/PI3K signaling, causing filopodia/lamellipodia to retract/internalize myelin debris. Indeed, Gal-3 knock-down leads to a sharp shift in microglial morphology from ameboid to branched-like, the rearrangement of actin filaments and the inactivation of cofilin, which then fails to trigger phagocytosis (Reichert and Rotshenker, 2019).

Recent work has demonstrated that overexpressing Gal-3 with electroporation in subventricular zone showed no inflammation in the healthy postnatal increased gliogenesis with an increased percentage of striatal astrocytes but a decreased percentage of OLG, mediated by an increased bone morphogenetic protein signaling (Al-Dalahmah et al., 2019).

Given that Gal-3 binds to multiple targets, its contrasting effects on OLG biology and myelination may reflect direct and indirect actions, i.e. those mediated by microglia, astrocytes or peripheral cells. Furthermore, the high heterogeneity and developmental and region-specific differences among microglia, astrocytes and OPC may have consequences in Gal-3 effects. Worth highlighting, our results show that an increase in intracellular Gal-3 levels does not produce changes in the expression of m-OLG marker MBP, and that only extracellular Gal-3 treatment accelerates OLG differentiation (Pasquini et al., 2011). In addition, this prodifferentiating effect is observed within a certain range of rGal-3 concentrations, above which the viability of the OLG is impaired, and within a specific time window along the oligodendroglial lineage (Pasquini et al., 2011; Thomas and Pasquini, 2019, 2020). Moreover, the biological activity of Gal-3 depends on metalloprotease cleavage and the interaction with mediators of the extracellular matrix (Pasquini et al., 2011; Thomas and Pasquini, 2018). Therefore, studies into the therapeutic potential of Gal-3 in demyelinating diseases should consider the delivery of rGal-3 to OPC, for example, through nanocarriers and exosomes able to cross the blood-brain barrier and targeted with specific antibodies such as anti-neural/glial antigen 2 or anti-PDGFRα. Exosomes have recently emerged as possible biomarkers and therapeutic agents in CNS disease. Moreover, exosomes pose therapeutic advantages, as they can deliver cargo to other cells, easily cross the blood-brain barrier and exhibit low immunogenicity (Thomas and Pasquini, 2018). The fact that Gal-3 can be excreted through exosomes thus paves the way for studies into the therapeutic potential and biomarker capacity of exosomal Gal-3. Alternatively, microglia should also be considered as a potential target, as Gal-3 induces an increase in their phagocytic capacity and fosters the clearance of myelin debris produced by demyelination, a key step in the onset of remyelination.

Altogether, findings so far indicate that Gal-3 mediates the glial crosstalk and favors remyelination both by driving OLG differentiation and promoting a phagocytic microglial phenotype which facilitates the onset of remyelination. These results shed light on some of the mechanisms underlying Gal-3 action and open doors for the identification of new signaling pathways regulated by Gal-3 to control OLG proliferation and differentiation. This knowledge may allow the development of new targets in the design of future therapies for demyelinating diseases such as MS.

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