• Basic Research •

# Possible role of sialylation of retinal protein glycans in the regulation of electroretinogram response in mice

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## Abstract

**• AIM: To evaluate if the nature, degree and extent of Siaα2-3-/Siaα2-6-sialylation of retinal protein glycans plays a possible role in the development and regulation of electroretinogram response (ERG) in mice.**

**• METHODS: Proteins extracted, from retinae of postnatal day 2 (PN2), PN7, and PN14 wild type (wt) and retinal degeneration 1 (rd1) mice were quantified, labeled and used for lectin-microarray profiling with immobilized lectins which recognize a wide range of N-/O-glycans. Net fluorescence intensities of lectin-ligand complexes were measured and images of fluorescent lectin-microarrays were acquired. From the binding curves between each lectin and protein extracts from PN14 wt and PN14 rd1 mice retinae, the protein concentration was selected to determine optimum signal intensity for lectin-ligand binding. Mean±SEM values of proteins and fluorescence-intensities of lectin-ligand-complexes between 45 lectins and 36 protein extracts from wt and rd1 mice retinae were compared for significance of differences.**

**• RESULTS: Comparison of the progressive relative changes in the sialylated glycans of retinal proteins from wt and rd1 mice showed that Siaα2-3Galβ1-4GlcNAc-glycans (but not Siaα2-6-glycans) were detectable and quantifiable from the retinal-proteins of PN7 and PN14 wt and rd1 mice. Siaα2-3-sialylation of retinal-protein Gal/α-linked-Gal-glycans was significantly increased with age in PN7 and PN14 wt and less so in PN14 rd1 mice. Siaα2-3-/Siaα2-6-sialylation of retinal-protein Gal/α-linked-Gal-glycans was absent in PN2 wt and rd1 mice. Comparison of published ERG responses of wt and rd1 mice retinae with degree of Siaα2-3-sialylation of retinal-protein-glycans showed that PN2 wt and rd1 mice lack both the ERG response and Siaα2-3-/Siaα2-6-sialylation of retinal-protein Gal/α-linked-Gal-glycans; rd1 mice with relatively lower Siaα2-3-sialylation of retinal-protein Gal/α-linked-Gal-glycans showed aberrant ERG response; and wt mice with significantly higher Siaα2-3-sialylation of retinal-protein Gal/α-linked-Gal-glycans showed normal ERG response.**

**• CONCLUSION: Degree of Siaα2-3-sialylation of glycans possibly regulates ERG function in mice.**

**• KEYWORDS:** electroretinogram response; glycome; lectin microarray; mice retinae; retinal development and degeneration

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## INTRODUCTION

Rod photoreceptor cGMP phosphodiesterase type 6 (PDE-6), an effector enzyme in the retinal photo-transduction cascade, is activated by the active form of G-protein transducin to hydrolyze cGMP. Resulting decrease in cGMP levels closes cGMP-gated channels, transiently hyper-polarizes rod photoreceptors' (PR) plasma membrane (PM) and decreases the release of neurotransmitter (NT) glutamate at the PR synapse. Wild type (wt) mice show normal retinal architecture and electroretinogram response (ERG). Retinal degeneration 1 (rd1) mouse, an animal model of retinitis pigmentosa, is deficient in PDE-6 activity due to a mutation in the β-subunit of PDE-6 gene. Resulting increase in cGMP level opens cGMP-gated channels, activates $Ca^{2+}$-ion channels, prolongs release of glutamate and depolarizes PR membranes. Such PR degenerate rapidly and show aberrant ERG response[1-3]. Repertoire of glycans, decorating PM proteins varies during tissue development and degeneration[4-8]; influences neuronal-signaling, angiogenesis and inflammation by binding to *cis-/trans-* Siglecs and Galectins respectively which specifically bind to sialic acid (Sia) glycans and galatans[9-12].

**Nature, Biosynthesis and Function of Retinal Glycans**

According to the nature of linkage between glycans and amino acid residues of proteins, mammalian cell glycans are classified as O-/N-linked oligosaccharides which are linked respectively to hydroxyl group of Thr, Ser and amino group of Asn residues of glycoproteins (GP) (Figure 1a). Membrane glycoprotein

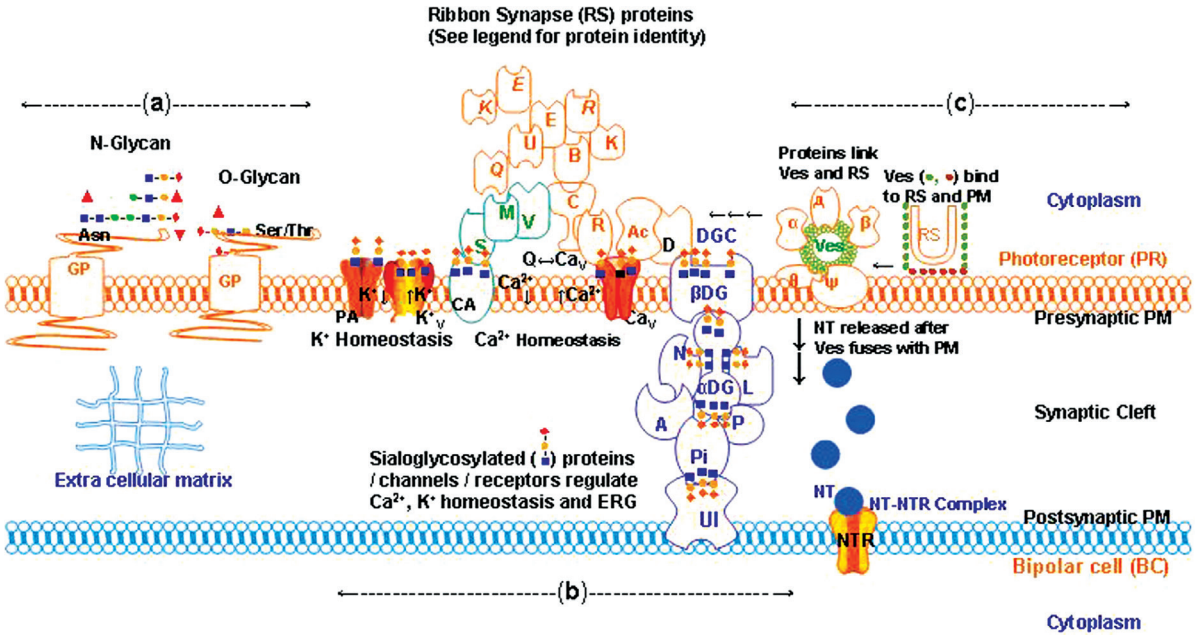


**Figure 1 Sialylation of retinal protein glycans possibly establishes synaptic junctions between PR- and bipolar-cells, and regulates retinal integrity, retinal function and ERG** Asn: Asparagine; BC: Bipolar cell; DGC: Dystrophin glycoprotein complex; ECM: Extracellular matrix; NTR: Neurotransmitter receptor; PR: Photoreceptor; PM: Plasma membrane; Ser: Serine; Thr: Threonine.

glycome shows cell/tissue type specificity, structural diversity, and dynamic quantitative changes during tissue development and degeneration. Diversity in the nature and linkages of glycome saccharides generates glycoprotein heterogeneity; which influences biological processes[13-17]. Sequence of saccharides in the glycome also encodes information for the conformation and spatial arrangement of glycoproteins in the PM[5-7,10]. Glycans displayed by glycoproteins modulate voltage-gated ion-channels, formation of synaptic-junction and release of NTs[17-21].

The arrangement of different GP and their glycans as well as of ribbon synapse (RS)/vesicles (Ves) in the PM, and of different families of proteins involved in RS function given in Figure 1 are as follows: namely a) GP anchored in the PM display Siaα2-3-sialylated (red-diamond, orange-circle, blue-square) N-glycans and O-glycans, Fuc (Fucose, red-triangle); man (mannose, green-circle); Gal (galactose, orange-circle); GalNAc (N-Acetyl galactosamine, orange-square); GlcNAc (N-Acetyl glucosamine, blue-square); Sia (red-diamond). b) Sialylated glycans displayed by GP $Ca^{2+}$ATPase (CA) voltage-gated $Ca^{2+}$ ion channel ($Ca_V$) and α,β-dystroglycan (α,β-DG) connect with the proteins Ac, D, Q, E, B, *E,* K, *R*, *K*, R, C, U, V, M, S, N, A, L, P, Pi and UI (see below for identity of proteins, red, green, blue color outlines) so as to participate in the generation of an ERG response. In absence of sialylated glycans the GP CA, $Ca_V$ and α,β DG are unable to maintain connectivity between glycoprotein-protein complexes especially through Ac, D, R, Pi and UI proteins which dampens the ERG response. Red, green and blue color outline represent different groups of proteins; Synaptophysin (α); Synaptobrevin (β); Complexin (д); SNAP25 synaptosome-associated 25 kDa protein (θ); Syntaxin (ψ); actin (Ac); dystrophin (D); $Ca^{2+}$ binding protein 4 (Q); Ribeye A (E); bassoon (B); Ribeye B (*E*)*;* piccolo (K); Rab3-interacting molecules (*R*); Kif3a kinesin family member 3a protein (*K*); RIM1 RIM2 Rab3-interacting molecules (R); CAST cytomatrix protein of the active zone of RS (C); Munc13 (U); Veli3 (V); MPP4 membrane palmitoylated protein 4 (M); PSD95 post synaptic density protein (S); neurexin (N); agrin (A); laminin (L); perlecan (P); pikachurin (Pi); unidentified (UI) protein; PA, $Ca^{2+}$-activated $K^+$-channel; $K_V$: $Ca^{2+}$ activated voltage-gated $K^+$ ion channel. Details of the identity, sialylation status and interactions between these proteins are from different reference number[16,22-25]. c) Horse shoe shaped RS is studded with lateral (olive-green-circle) and horizontal (mauve-circle) Ves which are filled with NT (blue-circle). Fusion of Ves to RS and the latter to pre-synaptic PM possibly involves proteins α, β, д, θ and ψ.

In mammalian cells, the nature of linkage and type and level of glucosylated-, galactosylated-, mannosylated-, fucosylated- and sialylated-glycans displayed by GP are influenced by a balance in the activities of the following pairs of enzymes namely glucosyltransferase/glucosidase, galactosyltransferase/galactosidase, mannosyltransferase/mannosidase, fucosyltransferase/fucosidase and sialyltransferase/sialidase, respectively. Sia, a signaling molecule, terminally masks the penultimate α2-3Gal-, α2-3GalNAc-, α2-6Gal- or α2-6GalNAc-glycan epitopes displayed by GP. Terminal Sia decorating neuronal receptors is recognized by *cis-/trans-*Siglecs and provides neuro-protection, modulates neural-

differentiation and -integrity during tissue development and degeneration[7,9-10]. Specific sialyltransferases link Siaα2,3- to β-D-Gal-glycans and Siaα2,6- to β-D-Gal/β-D-GalNAc/β-D-GlcNAc-glycans. Rodent retina shows high activities of sialyl-, fucosyl- and galactosyl-transferases and differentially expresses α2,3-sialyltransferases/α2,6-sialyltransferases during retinal development and degeneration. Decrease in the expression of sialyltransferases and/or increase in the activity of sialidases limit the number of terminally linked Sia residues leading to the unmasking of the penultimate β-Gal/β-D-GalNAc/β-D-GlcNAc-glycans. Unmasked GalGlcNAc, α1,2-fucosylated, but not the α1,3/4-fucosylated or α2,6-sialylated terminal glycans displayed by GP (namely laminin, fibronectin, integrin, transferrin and lysosomal membrane proteins) are recognized by *cis-/trans*- Galectins to influence angiogenesis and inflammation[26-31]. However, significance of the sialylated glycans in retinal biology is unknown and was therefore studied.

## MATERIALS AND METHODS

Details of the ethical approval, general methodology for progressive and relative quantification of the glycome by lectin microarray technique and for statistical analyses are the same as given in Ahuja[17]. Briefly, retinae from postnatal day 2 (PN2), PN7, and PN14 wt and rd1 mice (6 replicates each, total number 36) were dissected. Retinal proteins were extracted, quantified, labeled with Cy3 fluorescent dye, diluted (between 31.25 and 2000 ng·mL$^{-1}$) with Probing Solution (GP BioSciences Ltd., Yokohama, Japan). Diluted protein extracts were used (in triplicates) for lectin microarray profiling with 45 lectins immobilized on LecChip Ver 1.0 (GP BioSciences Ltd.). Net fluorescence intensities of lectin-ligand complexes were measured (in quadruplicate) and images of fluorescent lectin microarrays were acquired by using the evanescent-field fluorescence scanner (GlycoStation Reader 1200 GP BioSciences Ltd.). Results were analyzed after expanding the dynamic range of this data by gain merging method using GlycoStation Tools Pro Suite 1.5 (GP BioSciences Ltd.). From the binding curves between each of the 45 lectins and protein extracts (protein concentration between 31.25 and 2000 ng·mL$^{-1}$) from PN14 wt and PN14 rd1 mice retinae, protein concentration of 62.5 ng·mL$^{-1}$ was selected to determine optimum signal intensity for lectin-ligand binding. Mean±SEM values of proteins and fluorescence intensities of lectin-ligand complexes between 45 lectins and 36 protein extracts from PN2, PN7 and PN14 wt and PN2, PN7 and PN14 rd1 mice retinae[17] were compared for statistical significance of differences. One way ANOVA and Fisher's protected least significant differences; *post-hoc* comparisons were made (StatView Software, SAS, Chicago, IL, USA) and assigned significance as follows: $P\geq0.05$ non-significant; $P<0.05$ significant; $P\leq0.01$ very significant, $P\leq0.001$ highly significant. Forty five lectins used during this study specifically react with a wide range of N-/O-glycans displayed by proteins. Nomenclature, abbreviations, basic carbohydrate specificities and source of the lectins are as given by Hirabayashi *et al*[8].

## RESULTS

By using the lectin microarray technique dynamic and relative quantitative changes in the glycans of retinal proteins[17] were derived from the carbohydrate specificities of the lectins and levels of lectin-ligand complexes. For the first time a comprehensive repository of dynamic and quantitative global changes, in the nature and quantities of glycans representing PN2, PN7 and PN14 wt and rd1 mice retinal proteins, was prepared and published by Ahuja[17]. However, the significance of these changes in retinal biology could not be incorporated in this publication due to the large number and diversity of the glycans with different types of linkages and the same is described now.

Because of the interactions between Sia (a signaling molecule) and *cis-/trans*- Siglecs (receptor lectins for Sia) Sia influences patho-physiological processes[32]. Therefore, sialylation status (namely nature of linkages and extent of sialylation) of retinal glycans representing six different patho-physiological states of mice was selected from the repository of glycans referred above[17]. Changes in the sialylated glycan specificity of these lectin-ligand complexes have now been compared with the published ERG status of wt and rd1 mice retinae[1,3] so as to determine the role of Sia in the regulation of retinal function especially the ERG response[17].

### Significance of Siaα2-3-sialylation of Retinal Protein Glycans in the Regulation of Electroretinogram Response

From the analysis of the glycan specificity of lectin-ligand complexes it was evident that the unmasked Gal/GalNAc epitopes lacking Siaα2-3/Siaα2-6-sialylation were significantly increased (due to lower sialylation or higher desialylation) specifically in retinal proteins of rd1 mice[17]. And content of Fucα1-6GlcNAc (core Fuc)-, Fucα1-6GlcNAc- and Fucα1-6GlcNAc/Fucα1-3 (Galβ1-4) GlcNAc-glycans detected by the lectins AOL LCA and AAL (respectively from *Aspergillus orizae*, *Lens culinaris* and *Aleuria aurantia*) in retinal proteins of PN2, PN7 and PN14 wt ($P<0.05$ to $P\leq0.001$) and rd1 (*NS* to $P\leq0.01$) (except that by AAL) mice were significantly decreased with age. The decrease was lower in rd1 retinal protein glycans (due to lower sialylation or higher desialylation) as compared to those from wt mice. Fucα1-2Galβ1- or GalNAcβ1-glycans recognized by the lectin TJA-II (from *Tricosanthes japonica*) were detected only in rd1 retinal proteins especially those from PN7 rd1 retinae. Content of GalNAc-, Galβ1-4GlcNAc- and Galβ1-3GalNAc-glycans respectively detected by the lectins TxLC-I, RCA-120 and PNA (respectively from *Tulipa gesneriana, Ricinus communis* and *Arachis hypogaea*) formed a minor component but

were specifically higher in PN7 rd1 retinal proteins. Galβ1-4GalNAc-, α-linked terminal GalNAc-, Galβ1-3GalNAc/GalNAc-, and α-linked-Gal-glycans respectively detected by the lectins DSA, HPA, Jacalin and GSL-IB4 (respectively from *Datura stramonium, Helix pomatia, Artocarpus integliforia* and *Griffonia simplicifolia*) formed bulk of the galactosylated glycans. Galactosylated glycans recognized by the lectins DSA and HPA were decreased with age and the decrease was significantly ($P$<0.05) higher in the retinal proteins from wt mice (due to higher sialylation or lower desialylation) as compared to that in rd1 mice retinal proteins. The higher level of such non-sialylated glycans especially in the PN7 rd1 mice retinal proteins was apparently due to lower sialyltransferase or higher sialidase activities.

Siaα2-3Galβ1-4GlcNAc-glycans recognized by the lectin ACG (from *Agrocybe cylindraacea*) were detected in the retinal proteins both from PN7 and PN14 wt and rd1 mice but not in those from PN2 wt and rd1 mice. Siaα2-3Galβ1-4GlcNAc-glycan content of retinal proteins increased significantly with age both in the wt and rd1 mice but the increase was higher ($P$≤0.01 to $P$≤0.001) in PN7 and PN14 wt (due to higher sialylation or lower desialylation) as compared to that in the corresponding rd1 (NS to $P$≤0.01) mice retinae. Out of the total sialylated glycans (recognized by the lectin WGA, from *Triticum aestivum*), Siaα2-3Galβ1-4GlcNAc-glycan (recognized by the lectin ACG) constituted a relatively small fraction of the glycome of proteins of both wt and rd1 mice retinae. The higher level of sialylated glycans in wt mice retinal proteins is apparently due to higher sialyltransferase or lower sialidase activities. Siaα2-6Gal/GalNAc-glycans recognized by the lectins SNA, SSA and TJA-1 (respectively from *Sambucus nigra, Sambucus sieboldiana* and *Tricosanthes japonica*) and Siaα2-3Galβ1-4GlcNAc- and Siaα2-3Galβ1-3 (Siaα2-6) GalNAc-glycan recognized by the lectins MAL and MAH (from *Maackia amurensis*), respectively, were not detected in any of the protein extracts from PN2, PN7 and PN14 wt and rd1 mice retinae possibly due to low abundance of such sialylated proteins. PN2 wt and rd1 mice retinae do not show ERG response. However, with increasing age, ERG response develops normally in wt mice but is aberrant in rd1 mice retinae[1,3].

## DISCUSSION

Due to lack of appropriate technologies, the dynamically changing global profile, nature and quantity of glycans and their significance in retinal biology during retinal development and degeneration, has remained unexplored[23,33]. For such an evaluation lectin microarray technology became available only after the year 2010. By using the lectin microarray technique dynamic and relative quantitative changes in the glycans of PN2, PN7 and PN14 wt and rd1 mice retinal proteins[17] were derived from the carbohydrate specificities of the lectins and levels of lectin-ligand complexes. Changes in the sialylated glycan specificity of these lectin-ligand complexes[17] were compared with the published ERG status of wt and rd1 mice retinae[1,3] so as to determine the significance of Sia in the development and regulation of retinal function especially the ERG response. The possible role of Sialylated glycans associated with retinal proteins in establishing/regulating ERG function has now been explained here with respect to mice retinae.

Conventional synapse and RS are two basic classes of synaptic-junctions in mammalian retinae. At the conventional synapse between amacrine cells in the inner plexiform layer (IPL) and ganglion cells in the ganglion cell layer, NT release is triggered by brief bursts of action potential. At the RS between PR in the outer plexiform layer and bipolar cells (BC) in the IPL, NT is released continuously and the action potential change is gradual[22,28,34].

**Significance of Retinal Protein Glycans in the Regulation of Electroretinogram Response** Electrophysiological function of PR involves fusion of pre-/post-synaptic PM termini with horse shoe shaped sheets of excitatory RS studded with glutamate filled lateral and horizontal Ves (Figure 1c). Through sialylated glycans groups of proteins/GP in the RS connect PR cytoskeleton with the extracellular matrix leading to the ERG response (Figure 1b, 1c).

The proteins labeled as α, β, д, θ and ψ (Figure 1c), attach lateral Ves to the RS; and in a $Ca^{2+}$-ion dependent manner move Ves to horizontal position for fusion with the presynaptic PM of PR. Fusion of Ves to the pre-synaptic PM and release of glutamate is regulated by $Ca^{2+}$-ion homeostasis which is achieved by modulating the activities of $Ca^{2+}$-ATPase (CA) and voltage-gated $Ca^{2+}$-ion channel ($Ca_V$) (Figure 1b). Glutamate is then released for binding to the neurotransmitter receptor (NTR) anchored in post-synaptic PM of BC.

Through sialylated glycans, GP CA, voltage-gated $Ca^{2+}$-ion channel, α,β-DG of dystrophin glycoprotein complex (DGC), Ac, $Ca^{2+}$-binding protein 4 (Q) and dystrophin (D) interact with the RS proteins E, B, *E,* K, *R*, *K*, R, C, U, V, M, S, N, A, L, P, UI and Pi (for identity of the different groups of proteins see legends to Figure 1, red, green, blue outlines). Sialylated glycans displayed by a number of these proteins possibly form a bridge between PR and BC and establish cytoskeletal continuity[5-6] for the maintenance of retinal-structure[16,22], -function[12,23,28] and ERG response as described in different references[24-25,34-36].

**Significance of the Degree of Sialylation of Retinal Protein Glycans in the Development and Regulation of Electroretinogram Response** Lower degree of sialylation with Siaα2-3-/Siaα2-6- and higher proportion of unmasked galactosylated/fucosylated glycans displayed by rd1 mice retinal proteins suggests decreased sialyltransferase activity

and/or increased sialidase activity in rd1 mice retinae. Degenerating PR in rd1 mice retinae have been shown to up-regulate α-Klotho, an anti-aging protein[37]. α-Klotho has homology with sialidase/glycosidase for specific removal of Siaα2-6-residues to unmask and display GalGlcNAc-glycan (recognized by *cis-/trans-* Galectins) of $Ca_V$ and voltage-gated $K^+$-ion channel ($K_V$) in kidneys[38]. An imbalance in the activities of sialyltransferase and sialidase decreases sialylation of Gal/GalNAc-glycans of α-dystroglycan (α-DG)[13] which becomes unable to maintain connectivity with the proteins namely pikachurin (Pi), RIM2 (R), Ac, dystrophin (D) and an UI protein involved in the development of ERG response in mice[5-6,18]. ERG aberration in rd1 mice[1,3] could thus be attributed to the decrease in Siaα2-3-/Siaα2-6-sialylation of glycans by retinal sialidase activity. As reviewed above, GP $Ca_V$, CA, α,β-DG and $K_V$ have approximately 40% of their mass as glycans, of which ≥45% consists of Sia residues. And sialylated glycan based interactions between $Ca_V$, $K_V$ and CA, Pi, α,β-DG and some UI protein apparently regulate the ERG response in the wt and rd1 mice retinae.

It is concluded that: PN2 wt and rd1 mice lack Siaα2-3-/Siaα2-6-sialylation of retinal-protein Gal/α-linked-Gal-glycans and ERG response; rd1 mice with relatively lower Siaα2-3-sialylation of retinal-protein Gal/α-linked-Gal-glycans showed aberrant ERG response; and wt mice with significantly higher Siaα2-3-sialylation of retinal-protein Gal/α-linked-Gal-glycans showed normal ERG response. Targeting of Siglecs with Sia decorated nanoparticles has been shown to abrogate inflammation[32] and similar approach may be adopted for sialylation of desialylated RS proteins in mice retinae showing aberrated ERG function. These results suggest that extent of Siaα2-3-sialylation of retinal-protein Gal/α-linked-Gal-glycans possibly influence the development and maintenance of the ERG responses in mice.

Overall, the above findings suggest that lack or deficiency of Siaα2-3-sialylated Gal/α-linked-Gal-glycans and degree of Siaα2-3-sialylation of retinal protein glycans appears to have a possible regulatory role in the development and maintenance of the ERG function in mice retinae. As retinal proteins deficient in Sia show lack of protein-protein interaction[7,9-10,29], so sialylation pattern of retinal proteins serves as a hallmark of protein-protein interaction for ERG function, retinal integrity and health. These observations along with those in Ahuja[17] on retinal glycan profiles could open new avenues for diagnostic and therapeutic use in neurodegenerative diseases. By using TALENs or CRISPR-Cas9 programmable DNA editing gene therapy technologies, Li *et al*[39] restored dystrophin in iPSCs of Duchenne Muscular Dystrophy patients having a mutated dystrophin. However, dystrophin is a glycoprotein, also present in the mouse retina (Figure 1b) and possibly in other mammals as well. Restoration of dystrophin protein or other such proteins, without consideration for the extent of sialylation/glycosylation may not restore the function of glycosylated proteins consequently the efficacy of the gene editing therapy.

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**Conflicts of Interest: Ahuja S,** None.

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