LETTER TO EDITOR

Proteomic profiles of the retina in an experimental unilateral optic nerve transection: Roles of Müller cell activation

Dear Editor:

Retinal ganglion cell (RGC) degeneration is a common pathogenesis in multiple ocular disorders and was studied in animal reproducible models of optic nerve transection (ONT) for the neuronal apoptosis in the adult central nervous system.1-6 This directly affects RGCs as the main part of neuronal population apoptosis, although the exact molecular mechanisms remain unclear. The aim of our study is to explore proteomic profiles of retina in unilateral optic nerve transection and validate molecular interactions as new mechanisms. The “secondary degeneration” that RGC degeneration can be divided into two phases was hypothesized in acute and chronic eye diseases.7 The RGC axon damage resulted in intrinsic apoptosis triggered by activated retinal glial cells.8,9 Müller cells are the most abundant glial cells in the retina and function like astrocytes in the brain. The current study furthermore evaluates changes in protein expression in the retina and the function of Müller glial cells during RGC degeneration, and assesses potential effects on RGC damage. Our data provide new evidence for understanding secondary optic nerve injury and new targets for precise therapy.

We established and validated the rat ONT model, quantified the retinal protein profiles using iTRAQ, and compared the protein expression between ONT and Controls 1, 4, 7, 14, and 28 days after ONT inducion. About 4717 proteins were detected, of which 54 were deferentialy expressed proteins (DEPs) at five postoperative time points, including up-regulated 25 (>1.5-fold) and down-regulated 29 (<0.67-fold). About 708 DEPs were indentified within one postoperative time point (Figure S1). Figure 1A shows the top 10 up- and down-regulated proteins at each time point. Of those proteins, the expression of Alb, Mgarp and Scrn2 was significantly up-regulated, while Col1a1, Col1A4a1 and Dcn down-regulated (Table 1). DEPs were further classified into biological process, cellular component and molecular function proteins using bioinformatics analysis (Figure 1B). The 708 DEPs were classified into 236 pathways according to the KEGG pathway database. The most abundant pathways included metabolic pathway, ribosome, carbon metabolism, Huntington’s disease and spliceosome (Figure 1C). The protein–protein interactions of the 708 DEPs were grouped into six different clusters using the K-means method (Figure 1D). The critical nodules within interaction networks of DEPs contains Cdc5l, C3, Ppp2rla, and Optn. The top 10 up- and down-regulated DEPs was detected by hierarchical clustering at a time point/group after ONT induction (Figure 1E).

Expression of glial fibrillary acidic protein (GFAP) and complement component 1q (C1q) binding protein (gC1qR) increased, while GFAP over-expressed in acti-vated Müller cells after ONT-induced RGC denaturation. To furthermore validate the expression of GFAP and gC1qR sequentially upregulated proteins in the retina, we performed western blot (Wb) analysis on the retinas of Sprague Dawley (SD) rats with or without ONT induced RGC denaturation and confirmed the difference between (p < 0.05, Figure 2A). GFAP expression in the retina increased with the time after ONT and reached a peak at 14 days. Our results indicate that GFAP may be a disease-specific biomarker for activated Müller cells in the retina and GS a key enzyme in glutamate metabolism can be a biology-specific biomarker. Results from double immunofluorescence staining demonstrated that postONT GFAP and GS were co–localized at the end–foot and nerve fiber of Müller cells (Figure 2B, 2C).

Glutamate metabolic pathways are altered after retinal RGC injury. L-Glutamate/L-aspartate transporter (GLAST) is a critical glutamate transporter to effectively...
Proteomics and bioinformatics prediction. (A) The top 10 up-regulated and down-regulated differentially expressed proteins and fold changes in the retina of rats after optic nerve transection 1, 4, 7, 14, and 28 days after surgery. (B) Gene ontology (GO) analysis of 708 differentially expressed proteins detected in the study were categorised into the biological process (BP), cellular component (CC), and molecular function (MF). (C) The top 20 enriched KEGG pathways of differentially expressed proteins showed with bubble chart. (D) The protein-protein interaction of 708 differentially expressed proteins analyzed by STRING. The network was classified into 6 clusters by \( K \)-means method. The nodes represent protein in the network. (E) Hierarchical clustering analysis of the top 10 up-regulated and down-regulated differentially expressed proteins in the retina of rats after optic nerve transection at time points.
### TABLE 1

54 differentially expressed proteins (DEPs) were consecutively altered in the retina of rats after ONT at the five time points.

| Accession     | Protein name                                      | Gene symbol | 1 d after ONT | 4 d after ONT | 7 d after ONT | 14 d after ONT | 28 d after ONT |
|---------------|---------------------------------------------------|-------------|---------------|---------------|---------------|---------------|---------------|
| Q63156        | Decorin (Fragment)                                | Q63156      | .00223        | .01486        | .01536        | .02077        | .03293        |
| P02454        | Collagen alpha-1(I) chain                         | Col1a1      | .01369        | .02859        | .03236        | .03795        | .04426        |
| D3ZZT9        | Collagen type XIV alpha 1 chain                    | Col14a1     | .01469        | .02624        | .02420        | .03084        | .03978        |
| Q01129        | Decorin                                           | Dcn         | .02134        | .04279        | .01666        | .04150        | .01205        |
| F1LNH3        | Collagen type VI alpha 2 chain                     | Col1a2      | .02848        | .10143        | .09620        | .12839        | .09154        |
| A0A0G2KAI7    | Collagen alpha-1(XII) chain                        | Col12a1     | .05718        | .07436        | .06818        | .06733        | .07399        |
| Q07936        | Annexin A                                         | Anxa2       | .07961        | .11598        | .16246        | .10020        | .09331        |
| D3Z8V7        | Osteoglycin                                       | Ogn         | .09268        | .09347        | .11091        | .13388        | .15477        |
| P47853        | Biglycan                                          | Bgn         | .09383        | .07255        | .05176        | .11786        | .08047        |
| G3V8H7        | Olfactomedin-like 3                               | Olfm3       | .18007        | .18060        | .19388        | .19408        | .25997        |
| D3Z952        | Microfibril-associated protein 2                  | Mfap2       | .18127        | .38142        | .19513        | .27472        | .19324        |
| A0A096P6L8    | Fibronectin                                       | Fn1         | .18660        | .25948        | .22126        | .21145        | .24727        |
| B3Y9H3        | SI00 calcium binding protein A10                  | SI00A10     | .22431        | .26543        | .29485        | .24717        | .25176        |
| G3V8L3        | Lamin A                                           | Lmna        | .26497        | .31943        | .35378        | .32968        | .38461        |
| Q6P3E1        | Rps16 protein (Fragment)                           | RPS16       | .27947        | .37640        | .50116        | .58969        | .44949        |
| C0JPT7        | Filamin A                                         | Flna        | .32953        | .25454        | .49145        | .37053        | .34944        |
| Q5FVG5        | Tropomyosin 1                                     | TPM2        | .32989        | .28146        | .45933        | .43703        | .40284        |
| Q6MFZ1        | RT1 class 1                                       | RT1-M1-5    | .33476        | .31396        | .39337        | .38604        | .40935        |
| Q9P90         | Solute carrier family 22 member 17                | Slc22a17    | .34682        | .43023        | .32001        | .52572        | .37683        |
| Q8VIN2        | Annexin                                           | Q8VIN2      | .35220        | .64864        | .64505        | .34932        | .42196        |
| A0A0G2JWK7    | Transgelin                                        | Tagln       | .36476        | .40902        | .35256        | .37007        | .60992        |
| B2RZD4        | 60S ribosomal protein L34                          | Rpl34       | .37585        | .36258        | .45139        | .55468        | .41238        |
| A0A0G2K2V6    | Keratin, type 1 cytoskeletal 10                    | Krt10       | .38868        | .47179        | .18262        | .63517        | .34946        |
| A0A0G2K6J5    | Myosin light polypeptide 6                        | Myl6        | .43160        | .50325        | .66767        | .28007        | .50899        |
| Q9Z1P2        | Alpha-actin-1                                      | Actn1       | .45562        | .56864        | .47392        | .45700        | .49820        |
| G3V6P7        | Myosin, heavy polypeptide 9                        | Myh9        | .47272        | .33012        | .55756        | .53841        | .39933        |
| P68035        | Actin, alpha cardiac muscle 1                      | Actc1       | .47725        | .51444        | .54125        | .37805        | .48617        |
| P02680        | Fibrinogen gamma chain                            | Fgg         | .56785        | .39359        | .25029        | .33854        | .24229        |
| Q641Y0        | Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit | Ddost | .64635 | .42148 | .29379 | .39559 | .43517 |

(Continues)
remove excess glutamate from synaptic sites. We noticed that GLAST expression in rats without ONT was more widespread from the ganglion cell layer to the outer nuclear (ONL), especially in the outer plexiform layer. GLAST-labelled Muller cell processes were diffused throughout the ONL and GLAST expression increased from 7 days after ONT induction (Figure 3A). GLAST protein expression was significantly higher in rats with ONT group than those without ONT (Figure 3A, 3B).

We furthermore evaluated the changes in C1q expression in the retina, which is the ligand of gC1qR and the first element of the classic complement activation pathway. The interaction between C1q and gC1qR plays important roles in maintenance of the innate and acquired immunity and is closely associated with inflammation by initiating opsonization, amplifying recruiting phagocytes, and promoting membrane attack complex formation.10 In the normal retina, C1q is weakly expressed in RGCs and the inner and outer plexiform layers and obviously in Muller cell bodies and processes. We observed that C1q-labelled Muller cell processes were diffuse throughout the INL and ONL, and C1q increased significantly after ONT induction.

| Accession | Protein name | Gene symbol | 1 d after ONT | 4 d after ONT | 7 d after ONT | 14 d after ONT | 28 d after ONT |
|-----------|--------------|-------------|---------------|--------------|---------------|---------------|---------------|
| Q9QYU4    | Ketimine reductase mu-crystallin | Crym | 1.59123 | 1.53463 | 3.49471 | 2.48791 | 2.16249 |
| D3ZLZ7    | Inosine-5’-monophosphate dehydrogenase 1 | Impdh1 | 1.61002 | 2.52125 | 1.61227 | 1.82501 | 3.23548 |
| F1M471    | EPM2A-interacting protein 1 | Epm2aip1 | 1.62951 | 1.73340 | 1.71811 | 1.69181 | 1.87136 |
| Q5FVM4    | Non-POU domain-containing octamer-binding protein | Nono | 1.64205 | 2.29589 | 2.47585 | 2.0761 | 2.34220 |
| Q5XIE0    | Acidic leucine-rich nuclear phosphoprotein 32 family member E | Anp32e | 1.64668 | 2.20588 | 1.88964 | 2.28429 | 2.50702 |
| M0R3N4    | Vesicle amine transport 1-like | Vat1l | 1.69109 | 2.09379 | 3.01540 | 2.17249 | 2.13362 |
| A0A0G2JUX5 | Transcriptional activator protein Pur-beta | Pur-beta | 1.70230 | 2.80761 | 2.91012 | 2.05025 | 2.54624 |
| O88767    | Protein/nucleic acid deglycase DJ-1 | Park7 | 1.72324 | 2.70420 | 3.53109 | 2.25288 | 3.49859 |
| O35796    | Complement component 1 Q subcomponent-binding protein | Clqbp | 2.08203 | 1.84227 | 2.25983 | 1.74563 | 2.14898 |
| P47819    | Glial fibrillary acidic protein | Gfap | 1.73027 | 1.98764 | 6.60683 | 4.69927 | 4.92120 |
| P15887    | S-arrestin | Sag | 1.8691 | 2.35355 | 2.75898 | 5.41263 | 6.33378 |
| P12368    | cAMP-dependent protein kinase type II-alpha regulatory subunit | Prkar2a | 1.89630 | 2.57446 | 2.79823 | 2.25377 | 3.20962 |
| P1LNC8    | Interphotoreceptor matrix proteoglycan 2 | Impg2 | 1.92785 | 1.62596 | 3.58728 | 2.44987 | 3.02114 |
| P1LMW7    | Myristoylated alanine-rich C-kinase substrate | Marcks | 2.06343 | 2.14228 | 3.07641 | 1.97257 | 2.62340 |
| P04631    | Protein S100-B | S100b | 2.10259 | 2.42321 | 2.98445 | 3.07049 | 2.69259 |
| G3V6H9    | Nucleosome assembly protein 1-like 1 | Nap1/1 | 2.20634 | 3.55405 | 5.34293 | 3.81639 | 3.59727 |
| P07335    | Creatine kinase B-type | Ckb | 2.49786 | 2.38444 | 3.26172 | 2.45298 | 3.2785 |
| Q6AYR8    | Secernin-2 | Scrn2 | 3.10959 | 3.87169 | 2.94265 | 2.87996 | 2.74484 |
| D4A4W7    | Mitochondria-localized glutamic acid | Mgarp | 3.91372 | 4.41917 | 3.87734 | 3.13725 | 5.50719 |
| A0A1K0FUA6 | Globin a2 | LOC689064 | 4.92857 | 4.46899 | 2.17224 | 4.90831 | 3.48620 |
| A0A0G2JSH5 | Serum albumin | Alb | 6.19058 | 4.10714 | 4.22091 | 5.40322 | 5.35121 |
Expression and verification of GFAP, gC1qR and GS in the retina. (A) Glial fibrillary acidic protein (GFAP) and complement component 1q binding protein (gC1qR) in the retina after optic nerve transection at five time points were validated by western blot analysis and normalized to β-actin for semi-quantitative analysis. Data were represented into mean ± SD (n = 4). *p < .05, compared with control group. (B) Double immunofluorescence of glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) in the retina of rats after optic nerve transection (ONT). Retinal sections of the control group and the ONT group at days 7, 14, and 28 were stained with antibodies against GS (red) and GFAP (green). Nuclei were stained in blue using DAPI. (×400). (C) GS in the retina after optic nerve transection at five time points were analyzed by western blot and normalized to β-actin for semi-quantitative analysis. Data were represented into mean ± SD (n = 4). *p < .05, compared with control group. Scale bars = 25 μm. GCL: ganglion cell layer, INL: inner nuclear layer. ONL: outer nuclear layer (Figure 3C, 3D), as compared with Controls (p < 0.05). We also found that tumor necrosis factor (TNF)-α increased in the INL after ONT induction. TNF-α mainly expressed in Muller cells and that TNF-α protein expression was significantly higher by time after ONT than rats with ONT (p < 0.05; Figure 3E, 3F).

In conclusion, Müller cells were activated following ONT-induced RGC degeneration and accompanied by altered glutamate metabolism with the activation of classical complement pathways and inflammatory responses. Those reactions may contribute to RGC degeneration and provide new evidence to support the interaction between RGCs and Müller cells during primary and secondary RGC degeneration in the retina. Those alterations of key proteins can be a new class of targets for precision medicine therapy.

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FIGURE 3  Expression and verification of GS, GLAST, C1q, and TNF-α in retina. (A) Double immunofluorescence of glutamine synthetase (GS) and L-glutamate/L-aspartate transporter (GLAST) in the retina of rats after optic nerve transection (ONT). Retinal sections of the control group and the ONT group at days 7, 14, and 28 were stained with antibodies against GS (red) and GLAST (green). Nuclei were stained in blue using DAPI. (x400) (B) GLAST in the retina after optic nerve transection at five time points were analyzed by western blot and normalized to β-actin for semi-quantitative analysis. Data were represented into mean ± SD (n = 4). *p < .05, compared with control group. Scale bars = 25 μm. GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer. (C) Double immunofluorescence of glutamine synthetase (GS) and complement component 1q (C1q) in the retina of rats after optic nerve transection (ONT). Retinal sections of the control group and the ONT group at days 7, 14, and 28 were stained with antibodies against GS (red) and C1q (green). Nuclei were stained in blue using DAPI. (x400) (D) C1q in the retina after optic nerve transection at five time points were analyzed by western blot and normalized to β-actin for semi-quantitative analysis. Data were represented into mean ± SD (n = 4). *p < .05, compared with control group. Scale bars = 25 μm. GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer. (E) Double immunofluorescence of glutamine synthetase (GS) and tumour necrosis factor (TNF)-α in the retina of rats after optic nerve transection (ONT). Retinal sections of the control group and the ONT group at days 7, 14, and 28 were stained with antibodies against GS (red) and TNF-α (green). Nuclei were stained in blue using DAPI. (x400) (F) TNF-α in the retina after optic nerve transection at five time points were analyzed by western blot and normalized to β-actin for semi-quantitative analysis. Data were represented into mean ± SD (n = 4). *p < .05, compared with control group. Scale bars = 25 μm. GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer.
CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

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