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Up-regulation of DRP-3 long isoform during the induction of neural progenitor cells by glutamate treatment in the *ex vivo* rat retina

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ABSTRACT

Glutamate has been shown to induce neural progenitor cells in the adult vertebrate retina. However, protein dynamics during progenitor cell induction by glutamate are not fully understood. To identify specific proteins involved in the process, we employed two-dimensional electrophoresis-based proteomics on glutamate untreated and treated retinal *ex vivo* sections. Rat retinal tissues were incubated with 1 mM glutamate for 1 h, followed by incubation in glutamate-free media for a total of 24 h. Consistent with prior reports, it was found that mitotic cells appeared in the outer nuclear layer without any histological damage. Immunohistological evaluations and immunoblotting confirmed the emergence of neuronal progenitor cells in the mature retina treated with glutamate. Proteomic analysis revealed the up-regulation of dihydropyrimidinase-related protein 3 (DRP-3), DRP-2 and stress-induced-phosphoprotein 1 (STIP1) during neural progenitor cell induction by glutamate. Moreover, mRNA expression of DRP-3, especially, its long isoform, robustly increased in the treated retina compared to that in the untreated retina. These results may indicate that glutamate induces neural progenitor cells in the mature rat retina by up-regulating the proteins which mediate cell mitosis and neurite growth.

1. Introduction

Retinal regeneration presents therapeutic potential, offering hope for patients with visual loss due to retinal cell death. Despite neurogenesis continuously occurs in a few restricted areas of the brain, such as the sub-ventricular zone of the lateral ventricle and hippocampal dentate gyrus in the central nervous system (CNS) [1,2], adult retinal cells in mammals possess very limited ability of cell production. On the other hand, it has been shown that there are quiescent neural progenitor cells in the adult mammalian eye [3–6]. Activating factors for retinal progenitors have also been reported, including epidermal growth factor (EGF) [3,7], fibroblast growth factor 2 (FGF2) [3,6,7], insulin and insulin-like growth factor-1 (IGF-1) [8] and glutamate or its analogs [9].

In the CNS, glutamate is known not only as a major neurotransmitter [10] but also as a regulator of cell proliferation [11–13]. Although excessive activation of glutamatergic receptors causes excitotoxic cell death [14], interestingly, it has been shown that glutamate at low concentrations can induce retinal cell proliferation in higher vertebrates [9,15,16]. The protein dynamics during progenitor cell induction in the mature retina by glutamate still remain unknown. In this study we investigated specific proteins which act in rat retinal tissues when neural progenitor cells are induced by glutamate using two-dimensional electrophoresis (2-DE)-based proteomic analysis. We found that dihydropyrimidinase-related protein 3 (DRP-3), especially its isoform 2 (a.k.a. TUC-4b), was significantly up-regulated, not only at the protein level, but also in mRNA expression in the retina during retinal progenitor induction by glutamate.

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2. Materials and methods

2.1. Ethical statement for animal experimentation

The study was reviewed and approved by the Ethics Committee for Animal Experimentation of Yamaguchi University School of Medicine (Permit Number: 13-066). All experiments were carried out according to the Guidelines for Animal Experimentation of the Yamaguchi University School of Medicine and under the Law and Notification requirements of the Japanese Government. All efforts were made to minimize the suffering and number of animals used in this study.

2.2. Ex vivo rat retinal preparation

Male Sprague–Dawley rats (Chiyoda Kaihatsu Co., Ltd., Tokyo, Japan) at postnatal date (PND) 30 ± 2 were anesthetized and retinas were isolated and incubated using previously described methods [17].

2.3. Histology and immunocytochemistry

Retinal tissues were fixed and embedded in paraffin wax. The tissue block was cut into sections 4 μm thick. For light microscopic evaluation, the slides were stained with hematoxylin and eosin staining (Merck Millipore, Darmstadt, Germany). For immunohistochemistry staining, antigen retrieval was performed. The primary antibodies used were anti phospho-histone H3 monoclonal antibody (1:1000, #3377; Cell Signaling Technology, MA, USA), anti-nestin polyclonal antibody (1:100, N5413; Sigma–Aldrich, MO, USA) and anti-PAX6 monoclonal antibody (1:200, sc-53108; Santa Cruz, CA, USA). Confocal images were obtained using BZ-9000 series (BIOREVO) and BZ-II Viewer software (Keyence, Osaka, Japan).

2.4. Immunoblotting

Retinal tissues were homogenized in lysis buffer and immunoblots were performed as previously reported [18]. The primary antibodies used were anti-nestin polyclonal antibody (1:1000, N5413; Sigma–Aldrich), anti-PAX6 monoclonal antibody (1:200, sc-53108; Santa Cruz), anti-DRP-3 polyclonal antibody (1:500, 18969-1-AP; ProteinTech, IL, USA) and anti-actin (1:500, sc-1616; Santa Cruz).

2.5. Two-dimensional gel electrophoresis (2-DE)

Eighty microgram of protein was used for each 2-DE and isoelectric focusing (IEF) followed by SDS-PADE was performed as previously descrived [19]. The expression levels of the protein spots were quantified with Progenesis SameSpot software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). After statistical analysis, the selected spots whose expression was significantly different between the two groups were picked up for the MS analysis.

2.6. Immunoprecipitation assays

Immunoprecipitation was performed using a ProteinG Dynabeads kit (Dynal Biotech, Oslo, Norway). Fifty micrograms of denatured retinal lysate were incubated with 15 μl of anti-DRP-3 polyclonal antibody (ProteinTech). The target antigen was eluted and applied onto an SDS-PAGE gel. The gels were stained and the selected bands were excized from the gel for MS analysis.

2.7. In-gel digestion of proteins and MS analysis

The gel pieces were trypsinized to peptides as previously reported [20]. For MS analysis one of the following two systems was used: A liquid chromatography tandem mass spectrometry (LC-MS/MS) system (Agilent 1100 LC-MSD Trap XCT; Agilent Technologies, CA, USA). LC-ESI-MS/MS analysis using the QSTAR-Elite system (Applied Biosystems/AB SCIEX, Framingham, MA, USA) coupled with the Ultimate NanoLC system (Thermo Scientific, MA, USA) [20], [21].

2.8. Real time RT-PCR

Total RNA from the retina was isolated using RNeasy Mini Kit (Qiagen, Tokyo, Japan) and then the cDNA was obtained using a Reverse Transcription System (Promega, WI, USA). Real-time polymerase chain reaction (PCR) was performed using a LightCycler system (Roche, Basel, Switzerland). The sequences of the primer sets are shown in the supplementary material.

2.9. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Student's *t*-test was used for comparisons between two groups. For multiple comparisons, analysis of variance followed by a *post hoc* Holm–Sidak test was employed. Statistical analyses were performed using commercial software (SigmaStat 13.0; Systat Software Inc., San Jose, CA, USA). *P*-values of less than 0.05 were considered statistically significant.

Detailed descriptions of the methods are shown in the Supplementary material.

3. Results

3.1. Glutamate application to ex vivo retinal tissues induces mitotic cells in the ONL

We initially examined if glutamate application to *ex vivo* retinal tissues induced cell division. Control retinal sections (exposed to vehicle for 1 h, followed by incubation in bathing media for a total of 24 h) displayed no structural damage in any of the layers of the sensory retina (Fig. 1A). Retinal tissues incubated with 1 mM glutamate for 1 h, followed by incubation in glutamate-free media for a total of 24 h showed, consistent with prior reports [9,15], that glutamate induced the appearance of cells undergoing mitosis in the outer nuclear layer (ONL; the layer housing the nuclei of photoreceptor cells) without any histological damage (Fig. 1A). In order to confirm that these cells were really undergoing cell division, we stained retinal sections with phospho-histone H3 (pHisH3), a specific marker for cells undergoing mitosis [22]. We found that pHisH3 positive cells appeared in the ONL (Fig. 1A), with a statistically significant difference in the number of pHisH3 positive cells between the 0.3 mM and the 1 mM glutamate-treated groups ($P < 0.01$, $n = 5$, Fig. 1B). At a concentration of 3 mM the retinal sections exhibited excitotoxic damage (data not shown). Next we tested different incubation periods with 1 mM glutamate and found that 1 h is the minimum time required for inducing mitosis in our set-up (Fig. S1A and B). Thus in subsequent studies, the retinas were treated with 1 mM glutamate for 1 h prior to further analysis.

3.2. Glutamate induces neural progenitor cells in the mature retina

Next we examined whether the *ex vivo* retina treated with 1 mM glutamate for 1 h followed by incubation in glutamate-free media for a total of 24 h exhibited progenitor cells. When retinal sections were stained with an antibody against paired box 6 (PAX6), a

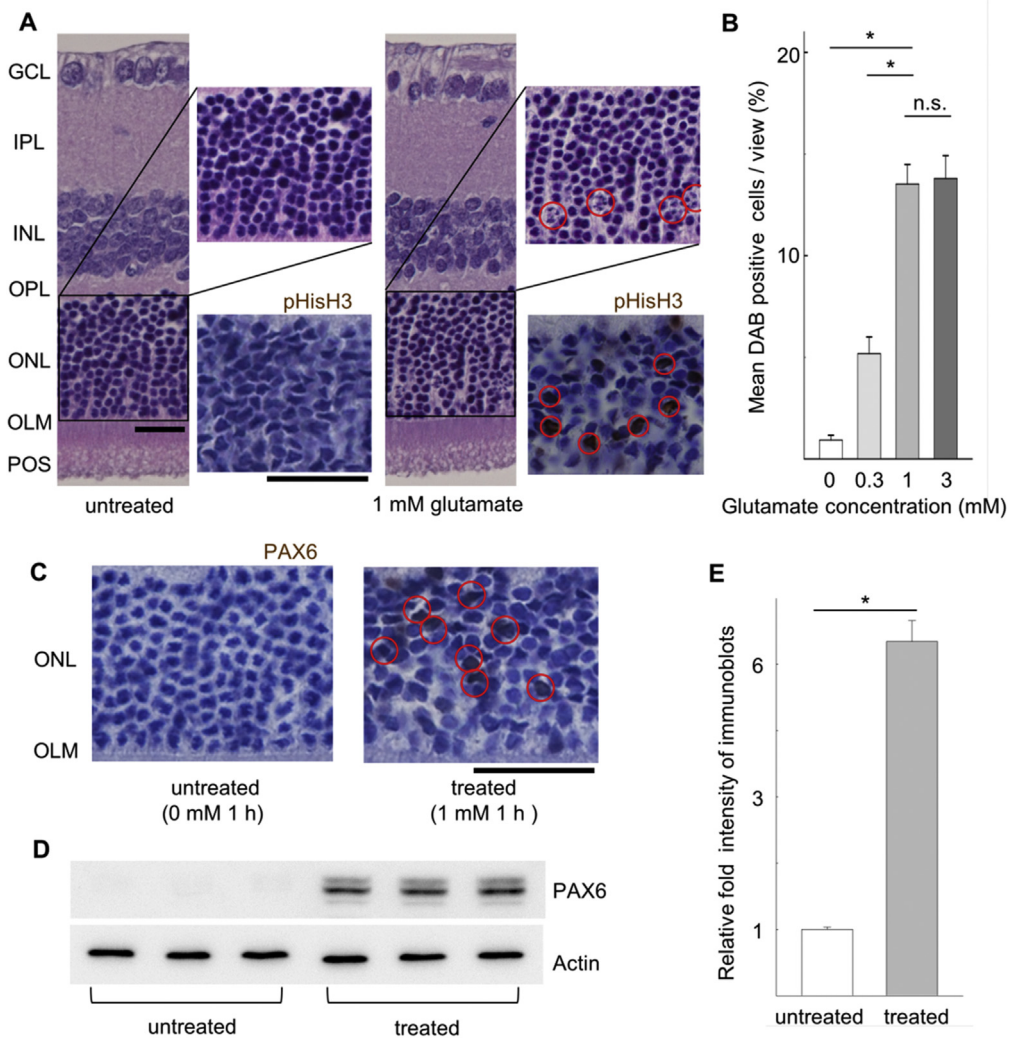


Fig. 1. Glutamate induces cells to divide and neural progenitor cells in the *ex vivo* retina. (A) The first panel shows a control retinal section with no structural changes in any of the layers including the ONL in the magnified view. A control section of the ONL showed very few phospho-histone H3 (pHisH3) positive cells, visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB). A retinal section incubated with 1 mM glutamate for 1 h, followed by incubation in glutamate-free media for a total of 24 h showed dividing cells in the ONL (red circles). Immunostaining revealed that pHisH3 positive cells arose in the ONL (red circles). (B) Summary of DAB positive cells at different concentrations of glutamate. Three fields were counted per slide using a 60X objective (n = 5). (C) Immunohistological staining with anti-PAX6 antibody and visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) in glutamate untreated and treated *ex vivo* retina. (D) Western blot analysis for PAX6 in glutamate untreated and treated retina. (E) Summary of immunoblot intensity for PAX6 in glutamate untreated and treated retina (n = 3). Data are mean ± standard error of the mean (SEM). Signals for protein levels were normalized to actin levels. *P*-values are calculated by Holm-Sidek *post-hoc* test. $^*P < 0.01$. Abbreviations: GCL, ganglion cell layer. IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OLM, outer limiting membrane; POS, photoreceptor outer segment; Scale bar: 20 μm.

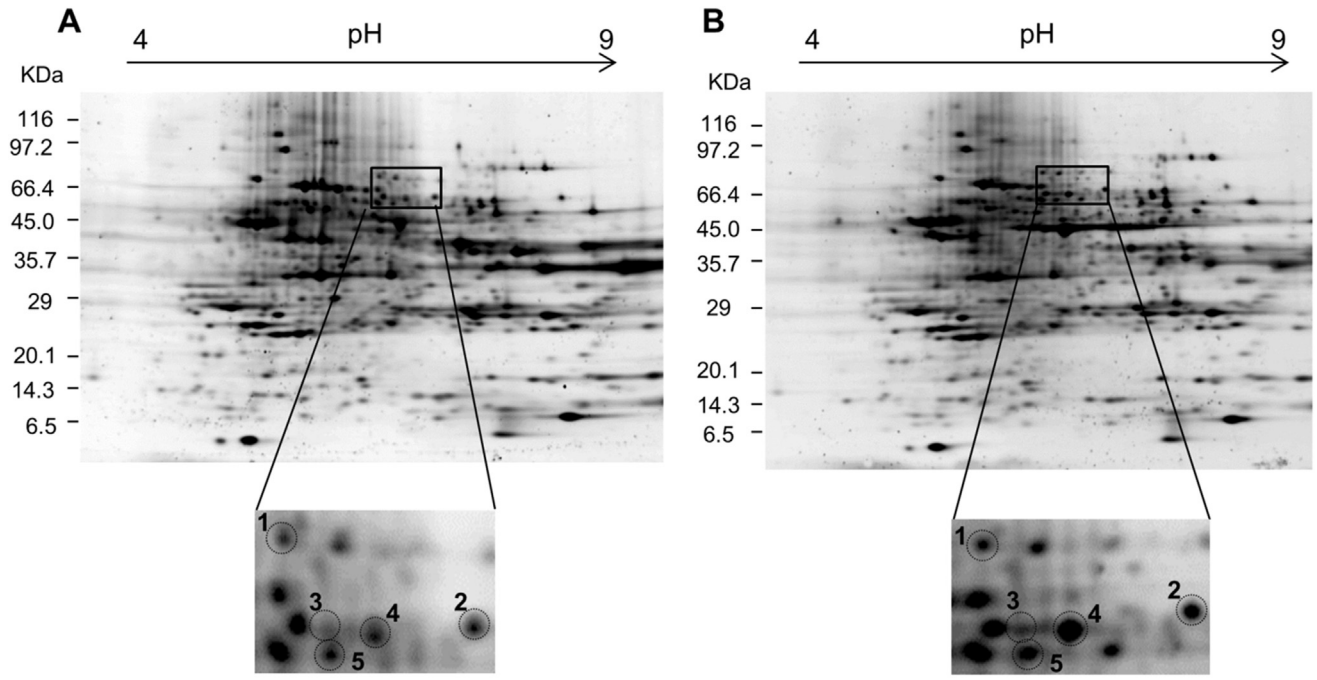
progenitor cell marker, which plays a pivotal role in eye development [23], we found that PAX6 appeared in the ONL (Fig. 1C). Immunoblotting confirmed a robust increase of PAX6 in the glutamate-treated retina (treated vs. untreated $P < 0.01$, n = 3, Fig. 1D and E). Similarly, a neural progenitor cell marker, nestin [9,24], also showed a substantial increase in the treated retina compared to the untreated retina (Fig. S2A). Consistent with the immunohistological evaluation, immunoblotting confirmed an increase in nestin in the treated retina (treated vs. untreated, $P < 0.01$, n = 3, Fig. S2B and C). Taken together, sub-toxic glutamate concentrations induced neural progenitor cells in the mature rat retina.

3.3. Proteins specifically induced by glutamate in the rat retina were identified by MS/MS

Since the protein dynamics of progenitor cell induction in the retina by glutamate have not yet been elucidated, we employed proteomic analysis on glutamate untreated and treated retinal sections. Retinal extracts from each group were subjected to 2DE and more than 1400 spots were separated in each group (Fig. 2A and B). The detected spots with significant difference in intensity were picked and subjected to mass spectrometry (MS). Identified proteins are listed in Fig. 2C. One of the reasons why only 3 proteins were identified with statistical significance in this study is that the cells induced by glutamate re-entering the cell cycle appeared almost exclusively in the ONL (Fig. 1) and this population is only a small percentage of the whole retina.

3.4. Immunoblotting for DRP-3 in rat retina following glutamate treatment

Dihydropyrimidinase-related proteins (DRPs) are cytosolic phosphoproteins that are highly expressed in the nervous system during development [25]. It has been reported that DRP-3 is



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Spot No.	Protein name	Accession No.	Theoretical *p*I	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in Glu groups (folds)
1	Dihydropyrimidinase-related protein 3	Q62952	6.04	61967.7	3	9	44.78	1.4
2	Dihydropyrimidinase-related protein 2	P47942	5.95	62277.9	4	11	52.43	1.8
3	Dihydropyrimidinase-related protein 2	P47942	5.95	62277.9	10	25	143.22	1.4
4	Stress-induced-phosphoprotein 1	O35814	6.40	62570.5	5	10	57.79	1.6
5	Dihydropyrimidinase-related protein 3	Q62952	6.04	61967.7	3	8	36.21	1.5

Fig. 2. Two-dimensional gel electrophoresis (2-DE) image of proteins from glutamate untreated (A) and treated retina (B) (n = 3). The spots with significant difference in intensity were subjected to mass spectrometry. (C) Identified proteins are listed. The spot number (circle) in A and B refers to the same number indicated in C.

expressed in the brain during the embryonic and early postnatal stages but very little in adults [26]. Consistent with this, the retinas from P30 rats in this study contained little DRP-3 (described as pre-treatment in Fig. 3A). In agreement with the observations from MS analysis, immunoblotting confirmed a significant increase of DRP-3 in the glutamate-treated retina compared to the non-treated retina (1.39-fold, $P < 0.01$, n = 5, Fig. 3B). Interestingly, DRP-3 bands of higher molecular weight in the treated retina tended to show greater intensity compared to those in the untreated group. More specifically, there was a 1.68-fold increase in the highest molecular weight band in the treated retina compared to the untreated retina ($P < 0.01$, n = 5, Fig. 3C). According to the molecular weight, the highest band matched DRP-3 isoform 2 [27].

3.5. MS analysis following immunoprecipitation using DRP-3 antibody on glutamate-treated samples confirmed the increased expression of DRP-3 isoform 2

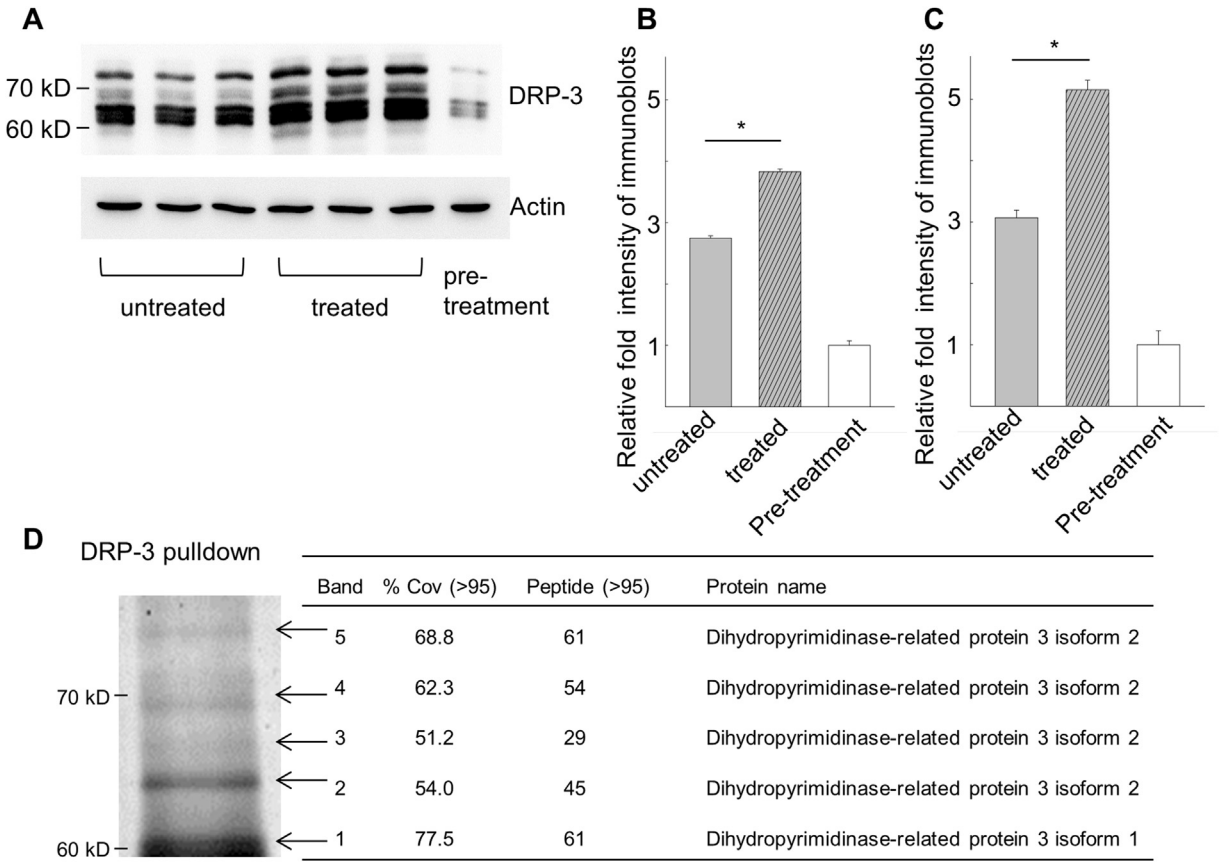
Since DRP-3 has two isoforms [27,28] and immunoblotting with DRP-3 antibody showed several bands, we assessed the results of the proteomic analysis using MS. We isolated proteins from the glutamate-treated retina by pull-down assay using DRP-3 antibody and the eluate was subjected to SDS-PAGE. After Coomassie Brilliant Blue (CBB) staining each stained band was excised and digested into peptides to determine the protein identity by MS. Each of the bands provided a reliable coverage of the DRP-3 peptide sequence (Fig. S3). Interestingly, 4 of 5 bands were identified as DRP-3 isoform 2 (Fig. 3D).

3.6. Glutamate-treated retina showed increased DRP-3 mRNA expression, especially for isoform 2

The MS analysis following the pull-down assay identified the higher bands of DRP-3, presenting greater intensity in the immunoblot (Fig. 3D), as DRP-3 isoform 2. This led us to examine the expression of DRP-3 mRNA in the retina following glutamate treatment. Total DRP-3 (isoform 1 + isoform 2) expression showed a significant increase in glutamate-treated retina compared to untreated retina ($P < 0.01$, n = 6, Fig. 4A). Surprisingly, DRP-3 isoform 2 expression in the treated retina increased more than 6 fold compared to that in the untreated retina (6.57-fold, $P < 0.01$, n = 3, Fig. 4B).

4. Discussion

In agreement with previous reports [9,16], glutamate induced neural progenitor cells in the mature retina. We explored protein dynamics during progenitor cell induction by glutamate in rat



Band	% Cov (>95)	Peptide (>95)	Protein name
5	68.8	61	Dihydropyrimidinase-related protein 3 isoform 2
4	62.3	54	Dihydropyrimidinase-related protein 3 isoform 2
3	51.2	29	Dihydropyrimidinase-related protein 3 isoform 2
2	54.0	45	Dihydropyrimidinase-related protein 3 isoform 2
1	77.5	61	Dihydropyrimidinase-related protein 3 isoform 1

Fig. 3. Immunoblot for DRP-3 and MS analysis following immunoprecipitation using DRP-3 antibody on glutamate-treated samples. (A) Western blot for DRP-3 in rat *ex vivo* retina. The "pre-treatment" sample was extracted from the retina immediately after dissection. (B) Summary of immunoblot intensity of all the DRP-3 bands in glutamate untreated, treated and pre-treated retina (n = 3). (C) Summary of immunoblot intensity of the highest molecular weight DRP-3 band (n = 3). (D) The isolated proteins from the glutamate-treated retina by pull-down assay using DRP-3 antibody were subjected to SDS-PAGE. Each stained band was digested into peptides to determine the protein identity by mass spectrometry (MS). Identified proteins with the highest coverage are listed (n = 5). Data are mean ± standard error of the mean (SEM). *P*-values are calculated by Holm-Sidek *post-hoc* test. $^{*}P < 0.01$. Western blot signals were normalized to actin levels.

ex vivo retina using 2-DE-based proteomics and found that DRP-2, DRP-3 and STIP1 protein levels were significantly increased. Notably, the mRNA expression of DRP-3 was also up-regulated, with its isoform 2 showing more than 6 fold increase compared to the control.

The DRP (TOAD64/Ulip/CRMP) family proteins are highly expressed in the developing nervous system and have been shown to be implicated in axon guidance and extension [29–31]. So far, five DRP members (DRP1-5) have been characterized in vertebrates [32,33]. It has been reported that DRP-3 is only expressed for a short period of time during the development of the CNS [25] and in the limited region of the adult hippocampus [34]. Consistent with this, little DRP-3 was expressed in mature rat retina in this study (Fig. 3). DRP-3 has been shown to regulate microtubule dynamics during mitosis [35] and bind to the stem cell marker CK15 in human adult breast epithelial progenitor cells [36]. These reports suggest that DRP-3 possibly plays a role in cell-cycle regulation during induction of neural progenitor cells in this study.

Each DRP member has two isoforms, a short isoform "a" (missing the N-terminal region) and a long isoform "b" [27,28]. However, the function of the long isoform of DRP-3 is not yet fully understood. Quinn and colleagues [27] have shown that DRP-3 isoform 2 regulates vesicular functions in the growth cone and promotes neurite extension and branching. Moreover, STIP1, known as a co-chaperon of the two major molecular chaperones, heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90) [37] was also confirmed to be up-regulated in glutamate-treated retina in this study (Fig. 2). Similar to the long isoform of DRP-3, STIP1 has also been reported to participate in modulating neurite outgrowth and neuronal protein synthesis in co-operation with prion proteins [38,39]. Together with STIP-1, DRP-3 isoform 2 may take part in mediating neuritogenesis in this study.

Glutamate is a major neurotransmitter in the CNS [10] and excessive glutamate is known to induce cell death, called "excitotoxicity" [14]. In other words, at a low concentration glutamate works as a mediator of excitatory signals to maintain brain function, however, at a very high concentration glutamate can be a trigger for lethal cell injury. On the other hand, glutamate at mid-range concentrations has been shown to have unique effects on neural progenitor cells, including controlling cell proliferation, survival and differentiation [40–42]. Actually, in this study, a sub-toxic concentration of glutamate induced neural progenitor cells in mature rat retina, as noted in Fig. 1. These unique effects by glutamate seem to mainly occur in the "sub-toxic" range between basal and toxic concentrations, however, the range may vary between species, tissues and age. Glutamate is also known to act through two receptors, ionotropic receptors and G-protein-coupled (metabotropic) receptors, and each receptor has been reportedly responsible for neural progenitor cell proliferation [13,41,42]. However, which sub-type of glutamate receptors is involved in our experiment still remains to be elucidated.

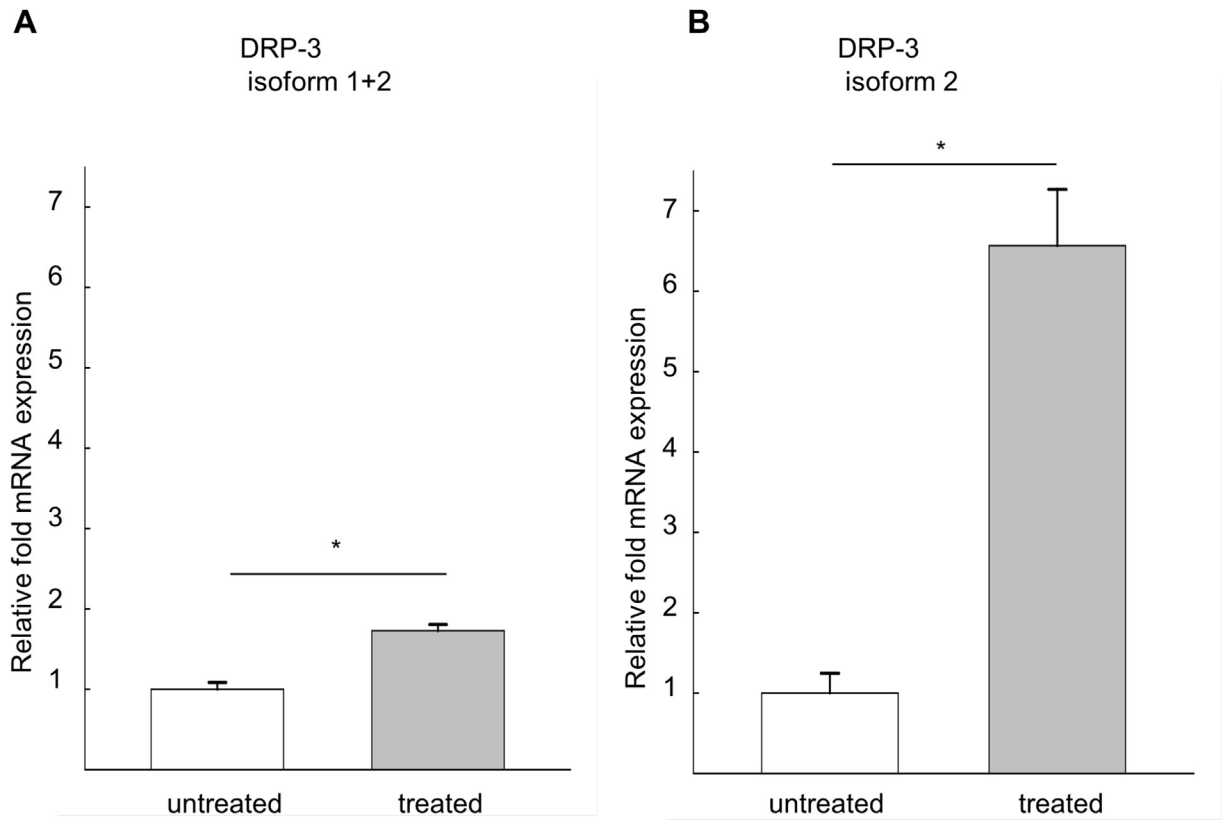


Fig. 4. DRP-3 expression in rat retina following glutamate treatment. Comparison of DRP-3 expression in glutamate-untreated and treated retina. (A) DRP-3 (isoform 1 + isoform 2) (n = 6). (B) DRP-3 isoform 2 (n = 3). Data are mean ± standard error of the mean (SEM). *P*-values are calculated by Student's *t*-test. $^{*}P < 0.01$ as compared to untreated.

In conclusion, we found the up-regulation of DRP-2, DRP-3 and STIP1 during neural progenitor cell induction by glutamate in *ex vivo* rat retina. Moreover, mRNA expression of DRP-3, especially, its isoform 2, robustly increased. Further investigations are required to elucidate how glutamate mediates DRP-3 expression and the function of DRP-3 isoform 2 in the induction of neural progenitor cells. Such studies will help the understanding of protein dynamics during progenitor cell induction by glutamate in the CNS, including the retina.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.102.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.102.

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