Glaucoma

Time-Dependent Effects of Reduced Cerebrospinal Fluid Pressure on Optic Nerve Retrograde Axonal Transport

Zheng Zhang,1–3 Shen Wu,1–3 Kegao Liu,1,2 Jingxue Zhang,1–3 Qian Liu,1–3 Lei Li,1 and Ningli Wang1–3

1Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing, China
2Beijing Ophthalmology and Visual Sciences Key Laboratory, Beijing, China
3Beijing Institute of Ophthalmology, Beijing, China

Correspondence: Ningli Wang, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, No. 1 Dongjiaominxiang Street, Dongcheng District, Beijing, 100730, China; wningli@vip.163.com.

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Mounting evidence from previous studies provides strong support for the concept that cerebrospinal fluid pressure (CSFP) in the subarachnoid space surrounding the optic nerve may have fundamental significance in the pathogenesis of glaucoma. A reduction in CSFP leads to an elevated pressure gradient across the lamina cribrosa (LC), predisposing the retinal ganglion cell (RGC) axons to damage at this site.1–10 However, the mechanism underlying optic nerve damage with the lowering of CSFP has not yet been fully elucidated.

Axonal transport is essential to ensure communication along the axons, and so the reported interruption of axonal transport has been considered to be an important pathogenic feature in glaucomatous optic neuropathy. Previous studies have demonstrated impairment of both anterograde axonal transport and retrograde axonal transport in the LC region after a rise in intraocular pressure (IOP).11–15 CSFP, as one of the two determinants of translaminar pressure difference (TLPD), should be included in discussions regarding the pathogenesis of glaucomatous optic neuropathy to enable inquiries into whether abnormally low CSFP is associated with abnormalities of axoplasmic flow. Using techniques with a reliable degree of reproducibility, we have previously observed a change in axonal transport in the rat optic nerve after 6 hours of CSFP reduction.16 Experimental models of elevated IOP have demonstrated a time-dependent change in axonal transport,17 but such a change has not previously been demonstrated in a reduced-CSFP model.

The purpose of this study was to determine whether the axonal transport change induced by reduced CSFP was dependent on the duration of the CSFP insult. Using an experimental model with IOP and CSFP monitoring, we studied axonal transport within the optic nerve after a brief period (1 hour or 3 hours) of CSFP reduction. The results of these experiments, taken together with those of our previous study16 on the effects of 6 hours of CSFP reduction, have allowed us to describe the temporal sequence of changes in axonal transport in the optic nerve after a reduction in CSFP. The study allowed us to investigate the vulnerability of axonal transport within the optic nerve after variable periods of CSFP reduction.
MATERIALS AND METHODS

General

Much of the methodology used in this study was identical to that of our previous report. Thirty-six animals were used for this work, and the experiments were performed on two separate groups. The first group consisted of 18 animals whose CSFP was reduced for 1 hour before euthanasia. The second group consisted of 18 animals whose CSFP was reduced for 3 hours before euthanasia. The data obtained in the group with 6 hours of CSFP reduction and the sham control group in our previously published study were also included in the present study. Eight-week-old adult male Sprague Dawley rats weighing 200 to 220 grams each were used for all experiments. The animals were kept in temperature-controlled rooms with a 12/12-hour light/dark cycle and were provided with standard food and water ad libitum. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Institutional Animal Care and Use Committee of Capital Medical University, Beijing.

Surgical Procedures

The surgical protocol was the same for both the 1-hour and 3-hour experiments. The rats were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). The head of the animal was positioned on a stereotactic guide instrument, and a midline scalp incision was made. The nuchal muscles were cut along the midline and stripped laterally to expose the transparent dura mater. The CSFP was measured using the BIOPAC Systems MP150 workstation (BIOPAC Systems Inc., Goleta, CA, USA). A dental drill (Dremel, Racine, WI, USA) was used to make a cannulation hole, 0.8 mm caudal and 1.5 mm lateral to the bregma. A 1.6F pressure catheter (4.8-mm tube length below the skull; Transonic Scisense, Inc., London, Ontario, Canada) was inserted vertically and advanced deep into the brain parenchyma. Using the BIOPAC Systems MP150 workstation, the CSFP, in millimeters of mercury, was continuously monitored. We then exposed the dura mater overlying the cerebellum and the dorsal surface of the medulla oblongata. A tapered glass capillary tube was inserted inside the cisterna magna under an operating microscope (Leica Microsystems, Wetzlar, Germany). CSF was cautiously aspirated from the cisterna magna every 15 minutes over a study period of either 1 hour or 3 hours. The dura mater incision was then closed with one or two interrupted 10-0 nylon sutures, and the muscles and the skin were sutured in layers with 4-0 nylon sutures. Although there could have been a continued leak from the CSF wound, it is likely to have been small. The IOP was measured in both eyes using a rebound tonometer that came factory calibrated for use in rats. Five consecutive IOP readings were averaged for each eye.

In order to assess retrograde axonal transport, the RGC axons were retrogradely labeled by injecting Fluoro-Gold (Biotium Inc., Hayward, CA, USA) into both superior colliculi at the same time points when the CSFP was reduced. The rats were deeply anesthetized with an intraperitoneal injection of 10% chloral hydrate in distilled water (4 ml/kg body weight) and positioned in a stereotactic apparatus. The skin over the cranium was incised, and the scalp was exposed. Holes approximately 2 mm in diameter were drilled into the skull on both sides of the midline, 6.2 mm posterior to the bregma and 1.5 mm lateral to the midline, with the dental drill before CSF aspiration. Using a 10-μl Hamilton syringe (Reno, NV, USA) with a 28-gauge needle, a total volume of 4.0 μl (2.0 μl per hole) of the axonal tracer dye hydroxyethylamidine (Fluoro-Gold 3% in saline) was microinjected sequentially into the left and right superior colliculi just at the beginning of CSF aspiration. The volume injected into each hole was delivered in two equal parts injected at two different levels, 4.0 and 4.2 mm below the pia mater. After each injection, the needle was left in place for 3 minutes to avoid reflux of the solution. The needle was then slowly withdrawn, and the skin was sutured closed. Fluoro-Gold is taken up by the axon terminals of the RGCs and transported retrogradely to the somas in the retina. The rats were removed from the stereotactic apparatus and allowed to recover from anesthesia, after which they were returned to their home cages and individually housed until sacrificed.

Tissue Preparation

The time at which CSF aspiration was begun was defined as the baseline. At 6 hours after baseline (low-CSFP-1h study group, n = 6; low-CSFP-3h study group, n = 6), at 1 day after baseline (low-CSFP-1h study group, n = 6; low-CSFP-3h study group, n = 6), and at 5 days after baseline (low-CSFP-1h study group, n = 6; low-CSFP-3h study group, n = 6), deeply anesthetized rats were transcardially perfused with 0.01-M PBS (pH 7.4) at 37°C, followed by 4% (wt/vol) paraformaldehyde. Because the injection time of the tracing compound was more synchronous with the CSF aspiration in the right eye, when the FG was injected into the left superior colliculi first, the retina of the right eye was examined in each rat. In order to explant the retina, a circular cut was made around the eye behind the ciliary body to separate the cornea and lens from the posterior portion of the eyeball. The retina was detached from the pigment epithelium and fully separated from the sclera by transection of the proximal optic nerve. The retinas were fixed in fresh 4% paraformaldehyde in PBS for 30 minutes and washed three times in PBS for 5 minutes each. The free-floating retinas were then flat mounted onto glass slides.

Axonal Transport

Axonal transport analysis was performed exactly as described in our previous report. In brief, RGCs were counted by blinded observers at a magnification of 200× under a Leica DM 400B fluorescence microscope. Fluoro-Gold-labeled ganglion cells were visualized under the following conditions: gain = 2.3×; saturation = 0.45; gamma = 2.76; and exposure times were 510.6 ms for animals killed at 6 hours after baseline, 212.2 ms for animals killed at 1 day after baseline, and 65.8 ms for tissue obtained at 5 days after baseline. Each retina was divided into superior, inferior, nasal, and temporal quadrants; three fields with a size of 800 × 600 μm were taken along the median line of each quadrant from the optic disc to the peripheral border of the retina at 1-mm intervals and were counted in a double-blind manner. The number of labeled cells was divided by the area of the region.
Postoperative CSFP 11.3 ± Baseline CSFP 11.6 ± Right eye IOP 12.8 ± 1.6

The mean preoperative CSFP across all rats was 11.3 ± 1.5 mm Hg. The mean postoperative CSFP was 2.2 ± 0.7 mm Hg for the low-CSFP group and 11.3 ± 1.6 mm Hg for the sham control group. The average differences between IOP and CSFP in the left and right eyes after surgery were 1.9 ± 1.6 mm Hg and 1.8 ± 2.0 mm Hg, respectively, across all rats. The average differences between IOP and CSFP in the left and right eyes after surgery were 10.8 ± 1.0 mm Hg and 10.9 ± 1.6 mm Hg, respectively, for the low-CSFP groups. Mean experimental data for the 1-hour, 3-hour, 6-hour, and sham control groups are presented in Table 1. Results from ANOVA testing revealed no significant differences in baseline CSFP, left-eye IOP, or right-eye IOP among the 1-hour, 3-hour, and 6-hour groups and the sham control group (one-way ANOVA; all P > 0.537). In the low-CSFP groups, the mean CSFP of 11.3 ± 1.5 mm Hg at baseline was reduced to 2.2 ± 0.7 mm Hg after the intervention. This result indicated a reduction in CSFP by approximately 81%. ANOVA revealed no significant differences in CSFP after surgery among the 1-hour, 3-hour, and 6-hour groups (one-way ANOVA; all P > 0.413).

### Statistical Analysis

All statistical testing was performed with SPSS Statistics 21.0 (IBM, Armonk, NY, USA). All results are expressed as the mean ± SD. The Kolmogorov–Smirnov test was applied to determine whether the data were normally distributed. Repeated-measures ANOVA with post hoc tests were performed to compare the relative RGC density among the 1-hour, 3-hour, and 6-hour low-CSFP groups and the sham group for the retrograde axonal transport assay. Normally distributed variables were analyzed using ANOVA with Bonferroni-corrected post hoc tests. Non-normally distributed variables were analyzed non-parametrically using ANOVA on ranks with a post hoc Tukey test for pairwise comparisons. A P value < 0.05 was considered statistically significant.

### RESULTS

#### Animal Physiology

In combination with the data obtained from the group with 6 hours of CSFP reduction and the sham control group in our previously published study, the findings in the present study included a total of 72 male Sprague Dawley rats. In 54 of the rats, CSF was cautiously aspirated from the cisterna magna for 1 hour (low-CSFP-1h study group, n = 18), 3 hours (low-CSFP-3h study group, n = 18), or 6 hours (low-CSFP-6h study group, n = 18). In 18 of the rats (sham control group), a trocar was positioned in the cisterna magna without removing the guide wire.

The mean preoperative CSFP across all rats was 11.3 ± 1.5 mm Hg. The mean postoperative CSFP was 2.2 ± 0.7 mm Hg for the low-CSFP group and 11.3 ± 1.6 mm Hg for the sham control group. The average differences between IOP and CSFP in the left and right eyes before surgery were 1.9 ± 1.6 mm Hg and 1.8 ± 2.0 mm Hg, respectively, across all rats. The average differences between IOP and CSFP in the left and right eyes after surgery were 10.8 ± 1.0 mm Hg and 10.9 ± 1.6 mm Hg, respectively, for the low-CSFP groups. Mean experimental data for the 1-hour, 3-hour, 6-hour, and sham control groups are presented in Table 1. Results from ANOVA testing revealed no significant differences in baseline CSFP, left-eye IOP, or right-eye IOP among the 1-hour, 3-hour, and 6-hour groups and the sham control group (one-way ANOVA; all P > 0.537). In the low-CSFP groups, the mean CSFP of 11.3 ± 1.5 mm Hg at baseline was reduced to 2.2 ± 0.7 mm Hg after the intervention. This result indicated a reduction in CSFP by approximately 81%. ANOVA revealed no significant differences in CSFP after surgery among the 1-hour, 3-hour, and 6-hour groups (one-way ANOVA; all P > 0.413).

### Axonal Transport

At 6 hours after the bilateral Fluoro-Gold injections into the superior colliculi, the density of RGC somas containing the fluorescent signal did not differ significantly between the low-CSFP-1h study group and the sham control group (all P > 0.912) (Table 2; Figs. 1A, 1D, 1F). At 6 hours after baseline, fluorescence was significantly lower in the low-CSFP-3h study group than in the sham control group (fluorescence reduced by 58% in the central region, 50% in the midperipheral region, and 44% in the peripheral regions; all P < 0.007) (Table 2; Figs. 1B, 1D, 1F). Fluorescence was significantly lower (by 86% in the central region, 81% in the midperipheral region, and 85% in the peripheral region) in the low-CSFP-6h study group than in the sham control group (all P < 0.001) (Table 2; Figs. 1C–1F). Compared with the low-CSFP-3h study group, the low-CSFP-6h study group showed a significant reduction in Fluoro-Gold-positive fluorescence (by 66% in the central region, 61% in the midperipheral region, and 72% in the peripheral areas; all P < 0.03) (Table 2; Figs. 1B, 1C, 1F).
Staining of retinal ganglion cells in retinal flat mounts after Fluoro-Gold was injected into both superior colliculi in the 1-hour (A), 3-hour (B), and 6-hour (C) study groups and in the control group (D). Images were obtained at 6 hours after baseline. (E) Quantitative analysis of the retrograde axonal transport assay. Error bars, SD; *significant at $P < 0.05$ when the low-CSFP-3h study group or the low-CSFP-6h study group was compared with the sham control group; $n = 6$ retinal flat mounts per group. Scale bars: 200 μm (A–D).

**Table 3.** Mean Density of RGCs Retrogradely Labeled with Fluoro-Gold at 24 Hours After Baseline

| Group          | n | Total   | Central Retinal Region | Middle Retinal Region | Peripheral Retinal Region |
|----------------|---|---------|------------------------|-----------------------|---------------------------|
| Low-CSFP       |   |         |                        |                       |                           |
| 1 h            | 6 | 1935 ± 217 | 2010 ± 345             | 2059 ± 301            | 1735 ± 164                |
| 3 h            | 6 | 1401 ± 141 | 1440 ± 144*            | 1511 ± 116            | 1252 ± 214                |
| 6 h            | 6 | 935 ± 256* | 913 ± 233*            | 1062 ± 279*          | 832 ± 351*                |
| Sham control   | 6 | 1696 ± 162 | 1885 ± 283            | 1714 ± 139            | 1490 ± 117                |

*Statistically significant at $P < 0.05$ when the low-CSFP-3h study group or the low-CSFP-6h study group was compared with the sham control group.
† Statistically significant at $P < 0.05$ when the low-CSFP-6h study group was compared with the low-CSFP-3h study group.

At 24 hours after baseline, the low-CSFP-3h study group, compared with the sham control group, showed a significant ($P = 0.047$) 24% reduction in Fluoro-Gold fluorescence specifically in the central region but showed no significant difference in fluorescence in the midperiphery or in the peripheral region (all $P > 0.112$) (Table 3; Figs. 2B, 2D, 2F). Fluorescence was significantly lower in the low-CSFP-6h study group than in the sham control group (fluorescence reduced by 52% in the central region, 38% in the midperipheral region, and 44% in the peripheral region; all $P < 0.027$) (Table 3; Figs. 2C, 2D, 2F). The low-CSFP-6h study group and the low-CSFP-3h study group differed significantly (all $P < 0.028$) in the density of Fluoro-Gold fluorescence-positive RGC somas.

At 5 days after baseline, the Fluoro-Gold fluorescence no longer differed significantly between the low-CSFP-3h study group and the sham control group (all $P > 0.096$) (Table 4; Figs. 3B, 3D, 3F). The low-CSFP-6h study group, compared with the sham control group, showed a significant ($P = 0.02$) 19% reduction in fluorescence, specifically in the peripheral region, and showed no significant difference in fluorescence in the central and midperiphery regions (all $P > 0.383$) (Table 4; Figs. 3C, 3D, 3F).

**DISCUSSION**

The time course of the change in axonal transport in the optic nerve after an acute transient reduction in CSFP has been described in this report. We compared the results from these experiments with those of our previous report to determine the time-dependent sequence of change. At 6 hours after the Fluoro-Gold injection, fluorescence in the retina was significantly lower in the low-CSFP-3h study group and the low-CSFP-6h study group, with no significant differences between the low-CSFP-1h study group and the sham control group. Retinal fluorescence was lowest in the low-CSFP-6h study group. At 24 hours after baseline, fluorescence no longer differed significantly between the low-CSFP-3h study group and the sham control group, and at 5 days after baseline the low-CSFP-6h study group and the sham control group no longer differed. The major finding from this study is that retrograde axonal transport change in the optic nerve occurs in a time-dependent pattern after a reduction in CSFP, with slower recovery of retrograde axonal transport following longer term CSFP reduction.

IOP and CSFP are important determinants of axonal transport in the optic nerve. Although previous investi-
FIGURE 2. Staining of retinal ganglion cells in retinal flat mounts after Fluoro-Gold was injected into both superior colliculi in the 1-hour (A), 3-hour (B), and 6-hour (C) study groups and in the control group (D). Images were obtained at 24 hours after baseline. (E) Quantitative analysis of the retrograde axonal transport assay. Error bars, SD; *significant at $P < 0.05$ when the low-CSFP-3h study group or the low-CSFP-6h study group was compared with the sham control group; $n = 6$ retinal flat mounts per group. Scale bars: 200 μm (A–D).

TABLE 4. Mean Density of RGCs Retrogradely Labeled with Fluoro-Gold at 5 Days After Baseline

| Group          | n | Total  | Central Retinal Region | Middle Retinal Region | Peripheral Retinal Region |
|----------------|---|--------|------------------------|-----------------------|--------------------------|
| LOW-CSFP       |   | Total  | Central Retinal Region | Middle Retinal Region | Peripheral Retinal Region |
| 1 h            | 6 | 2062 ± 217 | 2320 ± 212             | 2066 ± 230            | 1799 ± 230               |
| 3 h            | 6 | 1882 ± 135 | 2129 ± 120             | 1882 ± 173            | 1637 ± 147               |
| 6 h            | 6 | 1882 ± 189 | 2078 ± 279             | 2024 ± 112            | 1543 ± 212*              |
| Sham control   | 6 | 2089 ± 154 | 2315 ± 196             | 2045 ± 159            | 1906 ± 151               |

* Statistically significant at $P < 0.05$ when the low-CSFP-6h study group was compared with the sham control group.

FIGURE 3. Staining of retinal ganglion cells in retinal flat mounts after Fluoro-Gold was injected into both superior colliculi in the 1-hour (A), 3-hour (B), and 6-hour (C) study groups and in the control group (D). Images were obtained at 5 days after baseline. (E) Quantitative analysis of the retrograde axonal transport assay. Error bars, SD; *significant at $P < 0.05$ when the low-CSFP-6h study group was compared with the sham control group; $n = 6$ retinal flat mounts per group. Scale bars: 200 μm (A–D).
gators have studied axonal transport change after a rise in IOP\textsuperscript{12,15,19,20} or a rise in CSFP,\textsuperscript{21} few experiments have revealed any change in axonal transport after a reduction in CSFP. One possible reason is the difficulty of the surgery necessary for such an experimental design.

We did not find a significant difference in axonal transport between the control and low-CSFP-1h study groups; however, after 3 hours of CSFP reduction, the axonal transport of the low-CSFP eyes was significantly reduced in the central, midperipheral, and peripheral retinal regions. These findings, taken together with our previous report, suggest that measurable retardation of axonal transport in the rat optic nerve occurs after 1 to 3 hours of CSFP reduction, recovering when CSF is stopped from draining. The important difference was that after 6 hours of CSFP reduction, the reduction in Fluoro-Gold fluorescence was more severe and lasted longer. This implies that axonal transport retardation is sustained when CSFP is reduced.

Because of our experimental design, our study has been limited to the effects of an acute reduction in CSFP on axonal transport in the retrograde direction; however, as far as we are aware, this report is the first report to examine the temporal sequence of axonal change after a reduction in CSFP. Retrograde axonal transport is important for cell maintenance, such as the transport of vital trophic factors, including brain-derived neurotrophic factor. In the central nervous system, a decrease in these growth factors resulting from an alteration in retrograde trophic support has been thought to contribute to neuronal degeneration and prevent neuronal development.\textsuperscript{22} Through a similar mechanism, the inhibition of retrograde axonal transport in the optic nerve may play a role in the pathogenesis of RGC damage observed in diseases with reduced CSFP.

In recent years, a number of studies have suggested that reduced CSFP is a major risk factor for glaucomatous optic nerve axonal damage. Studying the time-dependent changes in axonal transport resulting from reduced CSFP provides a better understanding of the pathogenic role of reduced CSFP in RGC damage. Such time dependence also suggests that the period of CSFP reduction is critical in determining the severity of axonal damage. One of the limitations of this study is that the longest period of CSFP reduction was 6 hours. Although we have been able to report the effects of reduced CSFP on axonal transport change, it remains unclear whether there will be apoptotic cell loss in the RGC layer if we establish a rat model of longer duration CSFP reduction or repeated insults. The future study of chronic models of intracranial hypotension will further improve our understanding regarding the contribution of these changes to glaucomatous optic neuropathy. Another limitation of this work is that a rodent model of acute CSFP reduction was used. The rodent optic disc exhibits some histomorphometric differences from the human optic disc. The LC of the rat contains only sparse LC bundles that show no significant variation in laminar pore size.\textsuperscript{23} This is in contrast to the human LC, in which the laminar pores are larger within the superior and inferior regions than elsewhere.\textsuperscript{24} The optic nerve is affected asymmetrically in the early stages of human glaucoma, with the ganglion cell axons of the superior and inferior poles being the most susceptible to glaucoma damage.\textsuperscript{25} Because of these regional differences in laminar pore size, the effects of an acute rise in TLPD may result in a relatively selective and localized change in optic nerve axonal transport in human eyes that is different from the diffuse slowing axonal transport observed in the rodent optic nerve. This limitation could be addressed in experiments performed on primate models of cranial hypotension in which the LC structure has been shown to be more similar to the human structure.\textsuperscript{25} Our study does not suggest the potential mechanisms by which retrograde axonal transport is disrupted by lowered CSFP, and these should be further explored.

In conclusion, the axonal transport changes in the optic nerve occur in a time-dependent pattern after a reduction in CSFP; however, we should keep in mind that these changes in axonal transport are due to an acute but transient reduction in CSFP. The duration of this transient reduction in CSFP is relevant, as there is also a gap in time between when the acute reduction in CSFP ceases and when retrograde-labeled RGCs appear. Nevertheless, this finding may still be found to be important for improving our understanding of the pathogenesis of glaucomatous optic neuropathy.

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