Chapter 10
Axonal Transport

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Abstract Glaucomatous optic neuropathy is characterized by degeneration of retinal ganglion cell (RGC) axons and RGC death. Recent studies have suggested that glaucoma is a multifactorial disease that involves several molecular mechanisms. RGCs have long axons that provide spatial separation between the cell body and synapses. This anatomical structure has allowed us to use tracer injections to study axonal transport in RGCs. Increased intraocular pressure has been demonstrated to induce disturbances in axonal transport of tracers in RGCs. On the other hand, neurotrophic factors, particularly brain-derived neurotrophic factor (BDNF), have been demonstrated to support the survival of damaged RGCs in vitro and in vivo. Therefore, disturbances in retrograde axonal transport of BDNF may be associated with RGC death in glaucomatous optic neuropathy. Here we summarize the current understanding of axonal transport and BDNF in glaucomatous optic neuropathy. In addition, we introduce our approach to glaucomatous optic neuropathy, live imaging of axonal transport in RGCs, which may lead to the prediction of RGC death in glaucoma patients.

Keywords Axonal transport • BDNF • Live imaging

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10.1 Pathomechanisms of Glaucomatous Optic Neuropathy

Glaucomatous optic neuropathy is characterized by axonal degeneration in retinal ganglion cells (RGCs) and ultimately RGC death. In recent decades, research on glaucomatous optic neuropathy has made substantial progress and has indicated that glaucoma is a multifactorial disease.

In 1858, two hypotheses were proposed to explain pathomechanisms of glaucomatous optic neuropathy; these include the mechanical and vascular theories. The mechanical theory, which was proposed by Muller, hypothesized that increased intraocular pressure (IOP) compressed RGC axons and induced neuronal death. Approximately one million RGC axons project to the optic nerve head (ONH). Quigley et al. have used electron microscopy (EM) to evaluate human glaucomatous eyes and described several histological and structural findings [1]. First, they reported that an early increase in cup size preceded visual field losses and resulted from a loss of RGC axons. Second, they reported that as glaucomatous excavation increased in ONH, the backward and outward rotation of the laminar beams progressed. Finally, they reported that increased IOP led to a laminar distortion and subsequent compression of RGC axons.

On the other hand, the vascular theory, proposed by Von Jaeger, hypothesized that vascular dysfunction resulted in glaucoma. Interestingly, Kawasaki et al. have reported that retinal arteriolar narrowing is associated with a 10-year incidence of glaucoma [2].

In 1968, Lampert et al. proposed that interrupted axonal transport contributed to glaucomatous optic neuropathy [3]. Although this corroborates the mechanical theory, it does not exclude the vascular theory. Radius reported that in primates, occlusion of the short posterior ciliary circulation results in the interruption of axonal transport [4] and that the accumulation of a tracer of axonal transport was inversely proportional to the perfusion pressure [5].

10.2 Axonal Transport in Glaucomatous Optic Neuropathy

10.2.1 Basic Mechanisms of Axonal Transport

Neurons have complex structures that include axons. RGC axons are particularly long and must pass through the lamina cribrosa, which is vulnerable to IOP stress. Because neurons synthesize most proteins in the soma, they must send proteins to their synapses through axonal transport. The primary components of the neuron's cytoskeleton include microtubules, microfilaments, and neurofilaments. Especially, microtubules serve as the primary rail of axonal transport. Microtubules are made of α- and β-tubulin dimmers, which undergo polymerization and depolymerization. Microtubules have polarity that includes a fast-extending plus end and a slow-extending

minus end. Proximal dendrites include a mixed polarity of microtubules, whereas axons have a regular polarity with the plus end positioned toward the axon terminals. We have demonstrated that axonal transport of the brain-derived neurotrophic factor (BDNF) differs between axons and dendrites and these differences may result from the differences in microtubule polarity between dendrites and axons [6].

The quick-freeze, deep-etch EM method has helped to progress research on molecular motors. This method has revealed that molecular motors have a motor domain that connects with microtubules and a tail domain that connects with membranous vesicles. Kinesins and dyneins are responsible for microtubule-based axonal transport and use ATP for chemical energy. Most kinesins are responsible for anterograde axonal transport, which go toward the plus end of the microtubules. Most cytoplasmic dyneins are responsible for retrograde axonal transport, which go toward the minus end of the microtubules. Axonal transport is composed of fast transport and slow transport. The velocity of fast transport is 50–400 mm/day. By fast anterograde transport, synaptic vesicles, neurotransmitters, and mitochondria are mainly transported. By fast retrograde transport, endosomes and mitochondria are mainly transported. The velocity of slow transport is <8 mm/day. By slow transport, tubulins, actins, and neurofilaments are mainly transported. Slow transport is responsible for axonal maintenance and cytoskeleton repair. However, the molecular motors responsible for slow transport on microtubules have been not fully identified.

10.2.2 Studies of Axonal Transport in Glaucomatous Optic Neuropathy

Axonal transport in RGCs has been the focus of several glaucoma studies. RGCs are advantageous for studying axonal transport because they have long axons and their cell bodies and synapses are spatially separated. Anterograde axonal transport has been studied in RGCs using intravitreal tracer injections. Retrograde axonal transport of RGCs has been analyzed using tracers injected in central targets for RGC axons. The study by Anderson et al. indicated that axonal transport of tritiated leucine is blocked by an elevated IOP in the lamina cribrosa of primates [7]. Interestingly, Quigley et al. reported that by normalizing IOP after a 4 h period of increased IOP, leucine that had accumulated in the lamina cribrosa almost completely disappeared [8]. This reversal of axonal transport blockage suggests that axonal transport of leucine is disturbed in alive RGCs. In addition, this reversal suggests that RGCs with axonal transport disturbances may be a therapeutic target. Dandona et al. reported that in primates with chronic increased IOP, anterograde axonal transport from RGCs to the magnocellular layers in the dorsal lateral geniculate nucleus was less, compared with that to the parvocellular layers in the dorsal lateral geniculate nucleus [9]. Using immunohistochemistry, Pease et al. reported that BDNF and its tropomyosin-related kinase (TrkB) receptor had

accumulated in ONH of primates with chronic glaucoma [10]. Quigley et al. reported that acute increase in IOP in rats decreases retrograde transport of ^{125}I-BDNF from the superior colliculus [11]. Martin et al. reported that cytoplasmic dynein, which is responsible for fast retrograde axonal transport, accumulates in ONH with increased IOP in rats with experimental glaucoma [12]. This suggests that increased IOP could cease molecular motor movement. Buckingham et al. reported that retrograde axonal transport of tracers was impaired in RGCs long before axonal loss and neuronal death in DBA/2J mice [13]. Consistent with the findings reported by Buckingham et al. Salinas-Navarro et al. reported that the number of Brn-3-positive RGCs is greater, compared with the number of RGCs labeled by a retrograde axonal transport tracer 8 days after laser treatment [14]. This result suggests that in part of Brn-3-positive living RGCs, disturbances in retrograde axonal transport occur in mice with experimental glaucoma. Crish et al. reported that anterograde transport deficits of a tracer to the superior colliculus occurred and progressed in a distal-to-proximal manner in DBA/2J mice [15]. Moreover, they proposed that anterograde transport primarily decreases by aging, with increased IOP as an additional factor.

10.3 BDNF in Glaucomatous Optic Neuropathy

10.3.1 Basic Understanding of BDNF

However, how or whether axonal transport failure causes RGC death in adult mammals remains unknown. During retinal development, only RGCs that receive sufficient target-derived neurotrophic factors survive. This suggests that disrupted retrograde axonal transport of neurotrophic factors and neurotrophic factor deprivation induces RGC death in glaucomatous optic neuropathy. The neurotrophins are the most characterized family among neurotrophic factors. Neurotrophins, which are expressed in almost all neurons, play major roles in neurite growth, synaptic plasticity, and neuronal survival. In mammals, neurotrophins include nerve growth factor (NGF), BDNF, neurotrophin-3, and neurotrophin-4/5. In the early 1950s, Levi-Montalcini et al. [16] reported that the mouse sarcoma secretes a soluble factor that could stimulate growth of sensory and sympathetic neurons in chick embryos; this factor was later identified as NGF. In 1982, BDNF was purified from pig brain and was demonstrated to promote survival of chick sensory neurons [17]. BDNF binds to TrkB, a receptor in the tyrosine kinase family, and the p75 receptor. Because the first identified neurotrophin NGF is secreted from postsynaptic neurons and is retrogradely transported, BDNF was thought to be transported retrogradely alone. However, considerable evidence has indicated that BDNF is anterogradely transported. In 2001, Kohara et al. used BDNF tagged with green fluorescent protein (BDNF-GFP) to demonstrate that BDNF is anterogradely transported and transferred to postsynaptic neurons in an activity-dependent manner [18].

The BDNF gene includes five exons. After translation in ribosomes of the endoplasmic reticulum, the pre-pro-protein of BDNF is transported to the Golgi and the trans-Golgi network. BDNF is secreted by two pathways: the constitutive and regulated pathways. In the constitutive pathway, the prosequence of BDNF is cleaved by the trans-Golgi network-resident protein convertases. Vesicle release in the constitutive pathway does not rely on intracellular Ca^{2+}. In the regulated pathway, the prosequence of BDNF is cleaved after budding from the trans-Golgi network. Vesicle release in the regulated pathway is dependent on intracellular Ca^{2+}. We have demonstrated that BDNF is expressed in RGCs in a vesicular pattern in both axons and dendrites. BDNF vesicles produced in the neuron cell body are anterogradely transported to the synapse by the fast axonal transport. On the other hand, after BDNF that is released by postsynaptic neurons or via autocrine mechanisms binds to the TrkB or p75 receptor, the endocytotic vesicles are retrogradely transported.

10.3.2 BDNF in Glaucomatous Optic Neuropathy

Pearson reported that it takes several months for RGCs to be lost by selective degeneration of neurons in the lateral geniculate nucleus of adult cats using kainic acid [19]. This finding suggests that adult RGCs do not completely depend on neurotropic factors derived from their central targets. However, several studies have reported that BDNF promotes RGC survival. Johnson et al. reported that BDNF supports the survival of cultured RGCs in vitro [20]. Mansour–Robaey et al. reported that in rats with optic nerve transection, RGC survival (42 %) increases with intraocular injection of BDNF when compared with that (20 %) observed with an intraocular injection of vehicle 2 weeks after optic nerve transaction [21]. Interestingly, Cheng et al. [22] reported that in adult rats with optic nerve transection, the RGC survival rate (76 %) increased 2 weeks after optic nerve transection with TrkB gene transfer using adeno-associated virus combined with intravitreal injection of BDNF when compared with controls (10 %). Pease and Quigley proposed that retrograde axonal transport of BDNF is disturbed in glaucoma [10, 11]. Therefore, a disturbance of BDNF retrograde axonal transport in RGCs may be associated with RGC death in glaucomatous optic neuropathy.

10.4 Live Imaging of Axonal Transport in RGCs in Glaucomatous Optic Neuropathy

Recent studies on glaucoma suggest that glaucoma is a multifactorial disease that involves several molecular mechanisms and other types of retinal cells. Importantly, glaucoma is defined by degeneration of RGC axons and final RGC death. Here we introduce our approach for predicting RGC death in glaucoma [6]. We hypothesize that evaluating axonal transport in RGCs will allow us to detect

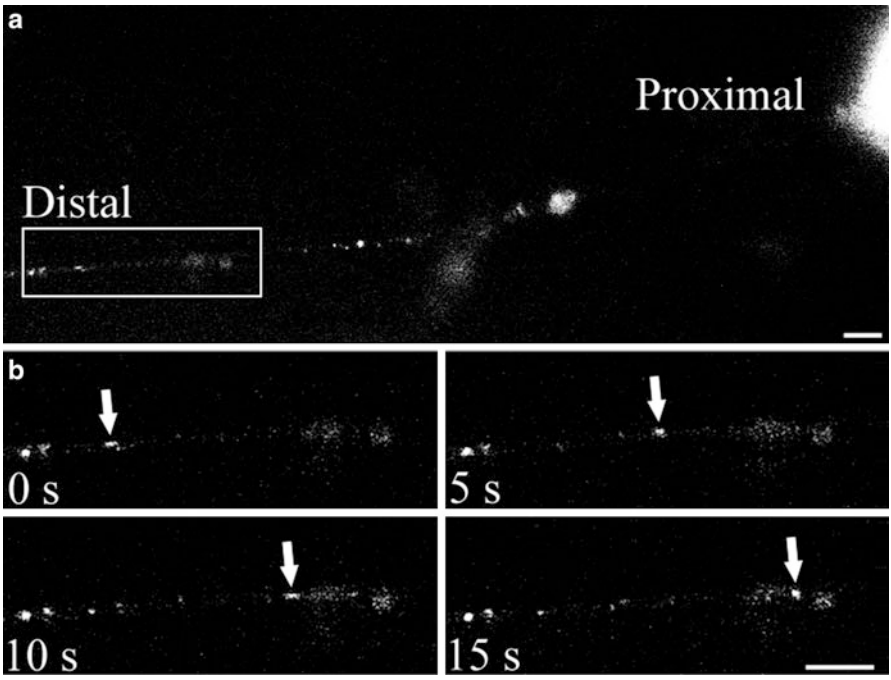

Fig. 10.1 Live imaging of retrograde axonal transport of BDNF-GFP in RGCs (**a**) RGC body is on the right side of Fig. 10.1a. (**b**) Magnification of Fig. 10.1a demonstrated a retrogradely transported BDNF-GFP vesicle. Scale bar, 5 μm. Data reproduced from Takihara et al. [6]

early axonal damage before RGC death in glaucoma. Interestingly, the data reported by Quigley et al. in a glaucoma model suggest that increased IOP disrupts axonal transport in alive RGCs and that by normalizing IOP after increased IOP, axonal transport blockage disappears before RGC death [8]. However, evaluation of axonal transport in RGCs has been limited to observations using fixed sections.

To visualize axonal transport of BDNF in living RGCs, we purified rat RGCs using a two-step immunopanning procedure and transfected purified RGCs with a plasmid encoding BDNF-GFP. To evaluate axonal transport of BDNF-GFP in living RGCs, time-lapse imaging was conducted in a chamber maintained at 37 °C and 5 % CO_2. Time-lapse imaging of the transfected RGCs revealed anterograde and retrograde axonal transport of BDNF-GFP (Figs. 10.1 and 10.2). To detect disturbances in axonal transport of BDNF-GFP vesicles resulting from axonal damage, we conducted time-lapse imaging of the transfected RGCs after treatment with colchicine, which disrupts the assembly of microtubules. The number of BDNF-GFP vesicles transported in RGC axons decreased 2 and 3 h after the colchicine treatment, as compared with controls (Fig. 10.3). BDNF-GFP vesicles which could not move smoothly were observed 3 h after the treatment (Fig. 10.3). Until 3 h after colchicine treatment, no RGCs were positive for

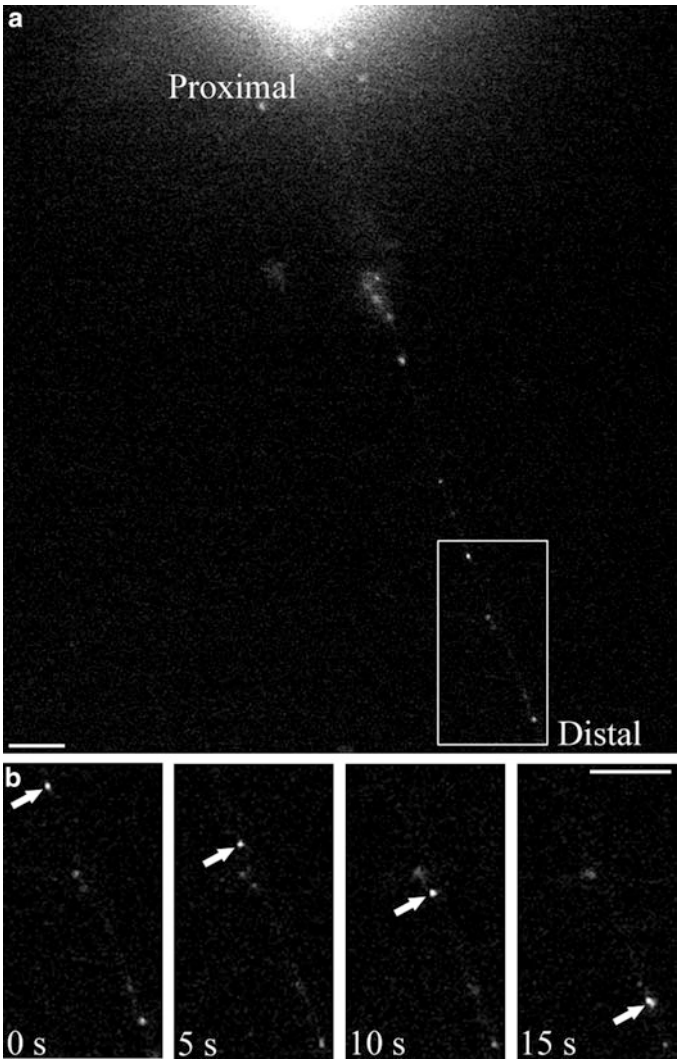

Fig. 10.2 Live imaging of anterograde axonal transport of BDNF-GFP in RGCs (**a**) RGC body is on the upper side of Fig. 10.2a. (**b**) Magnification of Fig. 10.2a demonstrated an anterogradely transported BDNF-GFP vesicle. Scale bar, 5 μm. Data reproduced from Takihara et al. [6]

ethidium homodimer-1. These results suggest that axonal transport of BDNF vesicles in damaged RGC axons is disturbed before RGC death.

Our approach may be useful in predicting RGC death in glaucoma patients. We believe that one problem in clinical practice with glaucoma patients is that IOP at which visual field loss does not progress differs among patients. In Japan, approximately 70 % patients with glaucoma have normal-tension glaucoma.

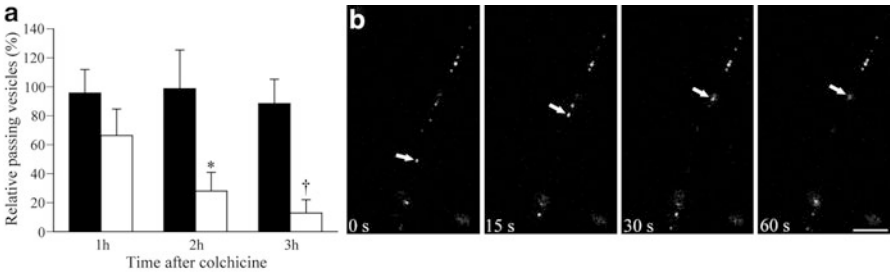

Fig. 10.3 Disturbance in axonal transport of BDNF-GFP after colchicine treatment (**a**) The number of BDNF-GFP vesicles significantly decreased 2 and 3 h after colchicine treatment (*$P = 0.003$, †$P = 0.0002$). (**b**) Representative time-lapse images of BDNF-GFP vesicles 3 h after colchicine treatment. The BDNF-GFP vesicle was not smoothly transported. Scale bar, 5 μm. Data reproduced from Takihara et al. [6]

We frequently experience patients whose visual field loss progresses even when IOP is less than 15 mmHg. The Collaborative Normal-Tension Glaucoma Study revealed that some patients experience a progression in visual field loss although their IOP was therapeutically decreased. In addition, these findings suggest that glaucoma is a group which has common glaucomatous optic neuropathy, but each of which have the heterogeneous main pathomechanisms. However, if we can quantify the stress level of RGCs with live imaging of axonal transport in RGCs, we may then be able to successfully predict RGC death in patients with glaucoma because the retina is the only part of the central nervous system that is directly visible without invasive techniques. Although there are many challenges in live imaging of axonal transport in RGCs of patients with glaucoma, the study of live imaging of axonal transport of BDNF in vitro suggests that live imaging of axonal transport in RGCs may be useful for predicting the future loss of RGCs in glaucoma and ultimately lead to the development of personalized medicine for patients with glaucoma.

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