ARTICLE IN PRESS

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Molecular Brain Research xx (2003) xxx–xxx

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/molbrainres

Short communication

Neurofilament subunits undergo more rapid translocation within retinas than in optic axons

Cheolwha Jung, Thomas B. Shea*

Center for Cellular Neurobiology and Neurodegeneration Research, Department of Biological Sciences, University of Massachusetts • Lowell, One University Avenue, Lowell, MA 01854, USA

Accepted 9 October 2003

Abstract

Axonal transport of neurofilaments (NFs) has long been considered to be regulated by phosphorylation, although recent studies have challenged this hypothesis. Our prior analyses of axonal transport in optic axons demonstrated two distinct NF transport rates that spatially and temporally correlated with changes in NF phosphorylation. In our prior studies, we focused on subunits already within axons. Re-examination of these data using additional approaches and examining additional earlier time points have allowed us to calculate rates at which subunits transport out of retinas and into optic axons. NF subunits were radiolabeled by intravitreal injection of ^{35}S-methionine. NF axonal transport was monitored by following the location of the front of radiolabeled subunits immunoprecipitated from retinas and segments of optic axons, which demonstrated four distinct transport rates. Subunits within retinas exhibited the fastest rate, and underwent a 50% slowing upon exiting the retina and entering optic axons. While this slowing could be due to a regional caliber increase and/or regional increase in NF phosphorylation within the first segment, prior studies indicated that inhibition of phosphatase activities increased NF phosphorylation within retinas and slowed NF subunit exit from retinas to a degree similar to that normally observed within the first segment of axons, suggesting that regional phosphorylation played a major role in slowing of NF transport following their exit from the retina. These findings provide additional support for the notion that phosphorylation regulates NF axonal transport.

© 2003 Published by Elsevier B.V.

Keywords: Neurofilaments; Axonal transport; Phosphorylation; Retina; Optic axons; Cytoskeleton

UNCORRECTED PROOF

Like all constituents of the axonal cytoskeleton, neurofilament (NF) proteins are synthesized within neuronal perikaryon and delivered to the axon by a process referred to as axonal transport [17,22]. NF transport slows progressively over time [7,9,14,18,34]. This slowing has generally been attributed to NF phosphorylation [1,3,7,9–13,16,20,30,34,36,40], although this continues to be debated [25,32]. A spatial and temporal correlation between increased C-terminal NF phosphorylation and slowing of NF axonal transport was observed in optic axons, revealing two distinct transport rates [9]. Herein, we demonstrate that NF subunits undergo transport at rates within retinas faster than those previously observed following their entry into optic axons.

Retinal ganglion cells were radiolabeled by injection of 70 μCi ^{35}S-methionine in 0.2 μl using a pulled glass capillary pipette into the vitreous [9,18]. Mice were sacrificed at intervals from 1 to 336 h following injection, retinas were dissected away from the rest of the eye and optic axons dissected into 9 × 1.1 mm segments. Retinas and segments from 11 mice were pooled and homogenized in 1% Triton X-100 in 50 mM Tris (pH 6.9) containing 2 mM EDTA, 1 mM PMSF and 50 μg/ml leupeptin at 4 °C by 50 strokes in a tight-fitting glass-Teflon homogenizer. The Triton-insoluble cytoskeleton was sedimented (15,000 × g for 15 min), resuspended in the above buffer without Triton, and NF subunits were immunoprecipitated from cytoskeletons by standard methods using a polyclonal antibody (R39) that quantitatively immunoprecipitates all three NF subunits, subjected to SDS-gel electrophoresis and autoradiography. Densitometric analyses of scanned autoradiographs was carried out with NIH Image. To compare subunit distribution at different post-injection intervals, total subunit radiolabeled recovered from the retina and all axonal segments was defined as 100% for each respective time interval. The relative amount of radiolabel in each segment was then

* Corresponding author. Tel.: +1-978-934-2881; fax: +1-978-934-3044.
E-mail address: Thomas_Shea@uml.edu (T.B. Shea).

0169-328X/$ - see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.molbrainres.2003.10.008

BRESM-90728; No of Pages 5

ARTICLE IN PRESS

expressed as a percentage of the total at that interval. Calculations were performed for individual autoradiographs from two separate experiments, and resultant values pooled. The transport rate of NF subunits was determined for the "front" of radioactivity, which was defined as that segment into which $\geq$ 10% of radiolabeled subunits had entered or traversed as utilized previously in this system [6,9–12]. The "average transport rate" for the front (where the front is considered to have traversed half of the segment in which it is recovered [6]) was calculated by adding 0.5 mm (i.e., approximately half the length of a segment) to the total distance of the 1.1 mm segments through which the front had migrated. Additional considerations were required to estimate transport rates within retinas. The most proximal portion of retinal ganglion cell axons is recovered within the retina itself. The length of these initial portions varies from 0.1 to 2.6 mm, with 55% >0.9 mm [19]. To exit the retina and enter segment 1 of the optic nerve, NF subunits must have traversed at least 0.1 mm of axonal length, and at most 2.6 mm. We therefore calculated axonal transport rates for NF subunits within retina based on these inter-retinal axonal lengths of 0.1, 0.9 and 2.6 mm versus the initial appearance of radiolabeled subunits within the first segment of the optic nerve. Autoradiographs of later time points (6–336 h) are reproduced with permission from our earlier study [9] to highlight the differences in subunit distribution over time, and to allow direct comparison of rates at 1 and 2 h after radiolabeling (which address transport within retinas) with rates observed at later time points (which address transport within axons [9–12]).

Radiolabeled subunits were recovered within segment 1 of optic axons within 1 h (Fig. 1). Assuming that these subunits have traversed the shortest (0.1 mm) inter-retinal axonal lengths, these subunits have undergone an average transport rate during this hour of 14.4 mm/day (Fig. 1; Table 1), which is at least fourfold faster than the fastest rate (3.4 mm/day) reported for transport of subunits within the optic nerve itself [9]. In addition, since 55% of the inter-retinal axons are >0.9 mm [19], some subunits may have transported at least 0.9 mm within this first hour, which would require substantially faster transport rates (Table 1).

Prior studies have demonstrated that retinas are virtually empty of radiolabel by 144 h [9], thus subunits within retinal ganglion cell bodies and within even the longest inter-retinal axons had either degraded or transported into at least segment 1 of optic axons by this time (see also Fig. 1). To address the potential contribution of subunit degradation, we compared the total amount of radiolabeled NF-L within retinas and optic axon segments at 1 and 144 h of radiolabeling. The total radiolabeled NF-L (retina + all axonal segments) following 144 h of radiolabeling (378.3 arbitrary densitometric units) exceeded that observed at 1 h following radiolabeling (240.0 arbitrary densitometric units) by >50%. This increase suggested that proteolytic degradation was

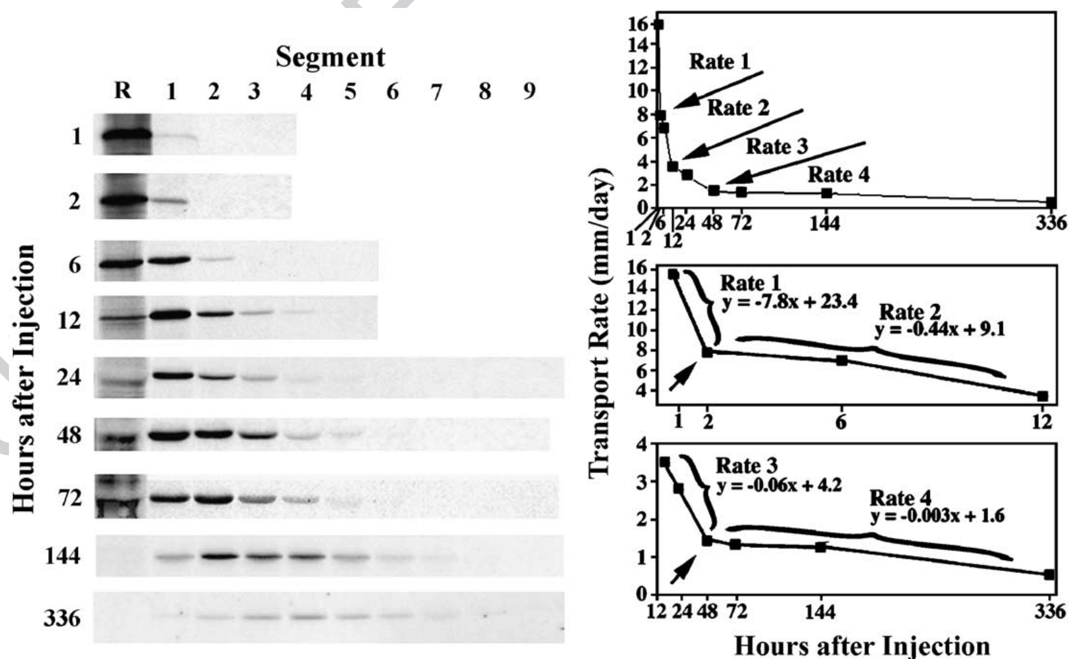

Fig. 1. Regional slowing of the transport rate of NFs in optic axon cytoskeletons. Panels present the region corresponding to NF-L from autoradiographs presented in this figure to facilitate comparison among samples. The accompanying graphs present the average transport rate (expressed in mm/day) of the front (the 10th percentile) of radiolabeled NF-L from 1 to 336 h calculated as described in the materials and methods part of this paper. Four major inflections in the curve of transport rate were noted, indicating regional slowings in NF transport rate (top graph; arrows denote transitions to slower rate). The lower two graphs present the intervals from 1 to 12 h, and from 12 to 336 h, to highlight the changes in transport rate; formulae derived by linear regression are presented for each of these distinct rates. Since the NF triplet co-migrated along optic axons, we present densitometric data only for NF-L for simplicity (e.g., Refs. [9,15]).

ARTICLE IN PRESS

Table 1
Axonal transport rates of radiolabeled NFs in optic axons

Location of front		Transport rate (mm/day)	
Time (h) after labeling	Axonal segment	0.1 mm axons	0.9 mm axons
1	1	14.4	33.6
2	1	7.2	16.8
6	2	6.8	8
12	2	3.4	4
24	3	2.8	3.5
48	3	1.4	1.75
72	4	1.3	1.57
144	5	1.25	0.97
336	7	0.51	0.57

Values represent the location of the front (as defined in the materials and methods part of this paper) as observed in the autoradiographs presented in Fig. 1. Average rates for 0.1 mm inter-retinal axons were calculated assuming that subunits have traveled 0.1 mm prior to exiting the retina, plus 1.1 mm for each optic axon segment that they have completely traversed, plus 0.5 mm (i.e., the midpoint) for that segment in which the front was recovered. The analogous calculations were made for 0.9 mm inter-retinal axons. These values were then multiplied or divided by the number of hours after labeling to derive a rate per day.

unlikely to account for the majority of depletion of radiolabeled NF subunits from retinas and instead reflects that continued synthesis and transport occurred during this time. In order for all radiolabeled subunits to have completely exited the retina and entered optic axons, some subunits must have traversed even the longest inter-retinal axons (2.6 mm). Utilizing this distance, the average transport rate (i.e., assuming that such subunits have migrated on average one-half of the distance for migration within segment 1 of optic axons) for the slowest-moving subunits exiting retinas can be estimated at 0.53 mm/day on average over the first 144 h. Notably, this rate exceeds that of the front of the moving wave of radiolabeled subunits at this time: at 144 h after radiolabeling, the front of the moving radiolabeled wave is recovered within segment 6 of optic axons (Fig. 1; Table 1). Assuming such subunits also traversed the longest intra-retinal axons, and assuming they have migrated half of the distance within segment 6, these subunits have traveled a total distance of 8.1 mm (2.6 + 5.5 mm for 5 × 1.1 mm optic axon segments + 0.5 mm for segment 6), indicating an average rate of 0.36 mm/day over this interval. Thus, even this conservative estimation of the time required for all subunits to undergo transport out of retinas reveals a transport rate nearly 50% faster than the average axonal transport rates for NF subunits further along optic axons at the same time period. These calculations further underscore that subunits undergo transport at faster rate within intra-retinal segments than within optic axons.

Increased axonal caliber also retards NF transport [8,23]. Subunits entering segment 1 encounter increased NF C-terminal phosphorylation [9,20], but also encounter the single major increase in axonal caliber 150 μm into the first axonal segment [4,5,7,20,27], each of which could therefore contribute to the regional slowing of NF transport as subunits exit from the retina. Manipulation of phosphatase activities suggests that regional phosphorylation plays a major role. Injection of the phosphatase inhibitor okadaic acid (OA) increased C-terminal NF phosphorylation within retinas and proximal axons and slowed NF transport out of retinas [9]. In these studies, the front of the moving wave had reached segment 3 within 1 day after radiolabeling, indicating an average transport rate of 2.8 mm/day over this 1 day interval (0.1 mm for intra-retinal axons + 2.2 mm for segments 1 and 2 + 0.5 mm for half of segment 3). However, following OA treatment, the front of the wave had not exited from segment 1, indicating an average transport rate of only 0.6 mm/day (0.1 mm for intra-retinal axons + 0.5 mm for half of segment 1), indicating that phosphatase inhibition induced at an approximate 60% decrease in the rate of transport of subunits out of the retina. This decrease is similar to the 50% decrease in transport rate observed under normal conditions between segments 1 and 2 (Table 1). Induction of a similar decrease in transport upstream of this caliber increase by phosphatase inhibition does not eliminate the potential contribution of the caliber increase to decreased NF transport, it is consistent with the notion that increased NF phosphorylation within segment 1 indeed contributes significantly to a regional slowing of NF axonal transport.

Diffusion of NF subunits is unlikely to account for a significant, if any, proportion of the rapid rate of translocation of subunits out of the retina, since only 11.1% of newly synthesized subunits departed from the retina within 24 h in the presence of OA, versus the 65.9% which did so in the absence of OA in 24 h [9]. While those subunits that were prevented from exiting the retina by OA must have required an active transport mechanism, it could be assumed that the 11.1% that did exit within 24 h did so by simple diffusion. Notably, however, we observed herein that >10% of newly radiolabeled subunits has entered segment 1 within 1 h; this rate is far in excess of the observation of a similar percentage within segment 1 over 24 h. In addition, there is a twofold bulk increase in NF subunits between retinas and proximal axonal regions [9]; any diffusion of radiolabeled NF subunits would have to be against a concentration gradient. Finally, nocodazole prevents the translocation into axons of NF subunits following their microinjection into perikarya [10], which further argues against diffusion providing any major contribution of diffusion to transport of NF subunits.

Our transport calculations presented herein remain conservative for at least two reasons. Since our initial observations were carried out at 1 h, by which time some subunits had already entered optic axons, we have not determined the transport rate of the most rapidly moving subunits. In addition, prior autradiographic analyses using exposure times up to 1 year demonstrated that NF subunits can undergo transport at markedly faster rates than those reported herein [14,15]. We did not utilize such long exposures herein, since they would over-expose retinal samples and thus preclude determination of exit of NF subunits from the retina [9].

By comparing the rate at which subunits exit the retina with rates along axons, and by monitoring translocation of the

UNCORRECTED PROOF

ARTICLE IN PRESS

front, rather than the peak [9,11], of the moving wave, we now document a total of four distinct transport rates for NFs within the optic pathway. Our results are in agreement with those presented for peripheral motor neurons [36], in which three distinct, progressively slower transport rates were observed for NFs. These authors suggested that different transport mechanisms might account for the three different rates. While this is possible, the demonstration that NFs can undergo fast axonal transport [26,33], and that the fast motors kinesin and dynein participate in NF axonal transport [24,28,35,37,38], also leaves open the possibility that differential association with a single motor system could mediate a range of transport rates [2,21,29]. Since phosphorylation regulates the association of NFs with kinesin [38], increased phosphorylation along optic axons [13,19,20] could generate progressive slowing of NF transport by generating progressively longer periods of dissociation of NFs from their motor(s) [14–16,38]. This possibility is supported by the observation that the slowest-moving NFs are the most highly phosphorylated [1,9,10,16,39]. Whether multiple motor systems contribute to the differential rates observed herein and in motor neurons [36] remains to be determined.

1. Uncited reference

[31]

Acknowledgements

This research was supported by the National Science Foundation.

References

[1] S. Ackerley, P. Thornhill, A.J. Grierson, J. Brownlees, B.H. Anderton, P.N. Leigh, C.E. Shaw, C.J. Miller, Neurofilament heavy chain side-arm phosphorylation regulates axonal transport of neurofilaments, J. Cell Biol. 161 (2003) 489–495.

[2] A. Brown, Axonal transport of membranous and nonmembranous cargoes: a unified perspective, J. Cell Biol. 160 (2003) 817–821.

[3] S.M. deWaegh, V.M-Y. Lee, S.T. Brady, Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells, Cell 68 (1992) 451–463.

[4] J.W. Griffin, P.N. Hoffman, A.W. Clark, P.T. Carroll, D.L. Price, Slow axonal transport of neurofilament proteins: impairment by b,b′ -iminodipropionitrile administration, Science 202 (1978) 633–635.

[5] J.W. Griffin, D.L. Price, P.N. Hoffman, Neurotoxic probes of the axonal cytoskeleton, Trends Neurosci. 6 (1983) 490–495.

[6] P.N. Hoffman, R.J. Lasek, J.W. Griffin, D.L. Price, Slowing of the axonal transport of neurofilament protein during development, J. Neurosci. 3 (1983) 1694–1700.

[7] P.N. Hoffman, J.W. Griffin, B.G. Gold, D.L. Price, Slowing of neurofilament transport and the radial growth of developing nerve fibers, J. Neurosci. 5 (1985) 2920–2929.

[8] J.-P. Julien, W.E. Mushynski, Neurofilaments in health and disease, Prog. Nucleic Acid Res. Mol. Biol. 61 (1999) 1–20.

[9] C. Jung, T.B. Shea, Regulation of neurofilament axonal transport by phosphorylation in optic axons in situ, Cell Motil. Cytoskelet. 42 (1999) 230–240.

[10] C. Jung, J.T. Yabe, F.-S. Wang, T.B. Shea, Neurofilaments undergo translocation into axonal neurites before incorporation into Triton-insoluble structures, Cell Motil. Cytoskelet. 40 (1998) 44–58.

[11] C. Jung, J.T. Yabe, T.B. Shea, C-terminal phosphorylation of the heavy molecular weight neurofilament subunit is inversely correlated with neurofilament axonal transport velocity, Brain Res. 856 (2000) 12–19.

[12] C. Jung, J.T. Yabe, S. Lee, T.B. Shea, Hypophosphorylated neurofilament subunits undergo axonal transport more rapidly than more extenisvely phosphorylated subunits in situ, Cell Motil. Cytoskelet. 47 (2000) 120–129.

[13] Y. Komiya, T. Tashiro, M. Kuokawa, Phosphorylation of neurofilament proteins during their axonal transport, Biomed. Res. 7 (1986) 345–348.

[14] R.J. Lasek, P. Paggi, M.J. Katz, Slow axonal transport mechanisms move neurofilaments relentlessly in mouse optic axons, J. Cell Biol. 117 (1992) 607–616.

[15] R.J. Lasek, P. Paggi, M.J. Katz, The maximum rate of neurofilament transport in axons: a view of molecular transport mechanisms continuously engaged, Brain Res. 616 (1993) 58–64.

[16] S.E. Lewis, R.A. Nixon, Multiple phosphorylated variants of the high molecular mass subunit of neurofilaments in axons of retinal cell neurons: characterization and evidence for their differential association with stationary and moving neurofilaments, J. Cell Biol. 107 (1988) 2689–2701.

[17] R.A. Nixon, The regulation of neurofilament protein dynamics by phosphorylation: clues to neurofibrillary pathobiology, Brain Pathol. 3 (1993) 29–38.

[18] R.A. Nixon, K.B. Logvinenko, Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed non-uniformly along axons of retinal ganglion cell neurons, J. Cell Biol. 102 (1986) 647–659.

[19] R.A. Nixon, S.E. Lewis, C.A. Marotta, Post-translational modification of neurofilament proteins by phosphate during axoplasmic transport in retinal ganglion cell neurons, J. Neurosci. 7 (1987) 1145–1158.

[20] R.A. Nixon, P.A. Paskevich, R.K. Sihag, C.Y. Thayer, Phosphorylation on carboxyl terminus domains of neurofilament proteins in retinal ganglion cell neurons in vivo: influences on regional neurofilament accumulation, interfilament spacing and axonal caliber, J. Cell Biol. 126 (1994) 1031–1046.

[21] S. Ochs, A unitary concept of axoplasmic transport based on the transport filament hypothesis, in: W.G. Bradley, et al (Ed.), Recent Advances in Myology, vol. 360, Excerpta Medica, Amsterdam, 1975, pp. 189–194.

[22] S. Okabe, N. Hirokawa, Dynamics of neuronal intermediate filaments, Mol. Biol. Cell 3 (1992) 355.

[23] H.C. Pant, H.C. Veeranna, Neurofilament phosphorylation, Biochem. Cell. Biol. 73 (1995) 575–592.

[24] V. Prahlad, B.T. Helfand, G.M. Langford, R.D. Vale, R.D. Goldman, Fast transport of neurofilament protein along microtubules in squid axoplasm, J. Cell Sci. 113 (2000) 3939–3946.

[25] M.V. Rao, M.L. Garcia, Y. Miyazaki, T. Gotow, A. Yuan, S. Mattina, C.M. Ward, N.A. Calcutt, Y. Uchiyama, R.A. Nixon, D.W. Cleveland, Gene replacement in mice reveals that the heavily phosphorylated tail of neurofilament heavy subunit does not affect axonal caliber or the transit of cargoes in slow axonal transport, J. Cell Biol. 158 (2002) 681–693.

[26] S. Roy, P. Coffee, G. Smith, R.K. Liem, S.T. Brady, M.M. Black, Neurofilaments are transported rapidly but intermittently in axons: implications for slow axonal transport, J. Neurosci. 20 (2000) 6461–6849.

[27] I. Sanchez, L. Hassinger, R.K. Sihag, D.W. Cleveland, P. Mohan, R.A. Nixon, Local control of neurofilament accumulation during radial growth of myelinating axons in vivo: selective role of site-specific phosphorylation, J. Cell Biol. 151 (2000) 1013–1024.

UNCORRECTED PROOF

[28] J.V. Shah, L.A. Flanagan, P.A. Janmey, J.F. Leterrier, Bidirectional translocation of neurofilaments along microtubules mediated in part by dynein/dynactin, Mol. Biol. Cell 11 (2000) 3495–3508.
[29] T.B. Shea, L. Flanagan, Kinesin, dynein and neurofilament transport, Trends Neurosci. 24 (2001) 644–648.
[30] T.B. Shea, P.A. Paskevich, M.L. Beermann, The protein phosphatase inhibitor okadaic acid increases axonal neurofilaments and neurite caliber, and decreases axonal microtubules in NB2a/d1 cells, J. Neurosci. Res. 35 (1993) 507–521.
[31] T.B. Shea, D. Dahl, R.A. Nixon, I. Fischer, Triton-soluble phosphovariants of the heavy neurofilament subunit in developing and mature mouse central nervous system, J. Neurosci. Res. 48 (1997) 515–523.
[32] T.B. Shea, C. Jung, H.C. Pant, Does C-terminal phosphorylation regulate neurofilament transport? Re-evaluation of recent data suggests that it does, Trends Neurosci.,(in press).
[33] L. Wang, C.L. Ho, D. Sun, R.K. Liem, A. Brown, Rapid movement of axonal neurofilaments interrupted by prolonged pauses, Nat. Cell Biol., (2000) 2137–2141.
[34] D.F. Watson, J.W. Griffin, K.P. Fittro, P.N. Hoffman, Phosphorylation-dependent immunoreactivity of neurofilaments increases during axonal maturation and b,b′ -iminodipropionitrile intoxication, J. Neurochem. 53 (1989) 1818–1829.
[35] C.H. Xia, E.A. Roberts, L.S. Her, X. Liu, D.S. Williams, D.W. Cleveland, L.S. Goldstein, Abnormal neurofilament transport caused by targeted disruption of neuronal kinesin heavy chain KIF5A, J. Cell Biol. 161 (2003) 55–66.
[36] Z. Xu, V.W. Tung, Temporal and spatial variations in slow axonal transport velocity along peripheral motoneuron axons, Neuroscience 102 (2001) 193–200.
[37] J.T. Yabe, A. Pimenta, T.B. Shea, Kinesin-mediated transport of neurofilament protein oligomers in growing axons, J. Cell Sci. 112 (1999) 3799–3814.
[38] J.T. Yabe, C. Jung, W.K.-H. Chan, T.B. Shea, Phospho-dependent association of neurofilament proteins with kinesin in situ, Cell Motil. Cytoskelet. 42 (2000) 230–240.
[39] J.T. Yabe, T. Chylinski, F.-S. Wang, A. Pimenta, S.D. Kattar, M.-D. Linsley, W.K.-H. Chan, T.B. Shea, Neurofilaments consist of distinct populations that can be distinguished by C-terminal phosphorylation, bundling and axonal transport rate in growing axonal neurites, J. Neurosci. 21 (2001) 2195–2205.
[40] B. Zhang, P. Tu, F. Abtahian, J.Q. Trojanowski, V.M.-Y. Lee, Neurofilaments and orthograde transport are reduced in ventral root axons of transgenic mice that express human SOD1 with a G93A mutation, J. Cell Biol. 139 (1997) 1307–1315.

UNCORRECTED PROOF