Polylysine Cross-Links Axoplasmic Neurofilaments Into Tight Bundles

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We have used axoplasm from the squid giant axon to investigate the effects of anionic and cationic polypeptides on the mobility and organization of axonal neurofilaments (NFs). Intact cylinders of axoplasm were extruded from squid giant axons into an excess volume of artificial axoplasm solution. In a previous study on the mobility of NFs in extruded axoplasm, we showed that these polymers disperse freely and diffusively into the surrounding solution, thereby expanding the axoplasmic cross-sectional area [Brown and Lasek, 1993: Cell Motil. Cytoskeleton 2Ck313-324]. In the present study, we found that 83nm-long ("long-chain") polylysine, a synthetic multivalent cationic protein, inhibited the radial expansion of isolated axoplasm and condensed the axoplasm, thereby reducing the cross-sectional area. Equivalent concentrations of a 7nm-long ("short-chain") polylysine did not inhibit the expansion of axoplasm and did not cause the axoplasm to condense. Inhibition of the expansion of axoplasm by long-chain polylysine was dependent on the polylysine concentration; condensation of axoplasm was observed at concentrations of 0.01 mg/ml (0.27 μM) or greater. Electron microscopy of the condensed axoplasm showed that the NFs were aligned side-by-side and in parallel in closely-packed bundles. Equivalent concentrations of 91nm-long ("long-chain") polyglutamate, a synthetic multivalent anionic protein, partially inhibited the expansion of axoplasm but did not cause the NFs to bundle and did not cause the axoplasm to condense. These studies indicate that cationic proteins bind tightly to the highly charged anionic surfaces of NFs and can link them together into compact bundles in a charge-dependent and length-dependent manner. The tightly packed organization of these cross-linked NFs differs from the normal loose organization of NFs in healthy axons. However, tightly bundled NFs are sometimes found in certain neuropathologies, such as giant axonal neuropathy.

Key words: neurofilament, axoplasm, axonal cytoskeleton, giant axon, squid

INTRODUCTION

Our laboratory has studied the organization of neurofilament (NF) populations in intact axons [Price et al., 1988, 1990, 1993; Monaco et al., 1989] and in isolated axoplasm [Brown and Lasek, 1993]. These studies have shown that neighboring NFs interact only weakly with one another and that the weak interactions between these polymers allow them to move from side-to-side by Brownian motion in response to the thermal forces that operate continually in cells at the molecular level. For our studies on NFs in isolated axoplasm, we extruded cylinders of axoplasm from squid giant axons directly into an excess volume of artificial axoplasm solution (AAS), thereby separating the axonal cytoplasm from the

Received June 28, 1994; accepted January 3, 1995.

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Abbreviations: AAS, artificial axoplasm solution; EGTA, ethylene-glycol-bis-[2-aminoethyl ether]-N,N,N',N'-tetraacetic acid; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; Mw, relative molecular mass; MT, microtubule; Mw, weight-average molecular weight; NF, neurofilament.

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confining of the plasma membrane that envelops it [Brown and Lasek, 1993]. The mobility of the NFs in these naked axoplasmic cylinders was then quantified using light and electron microscopy. Light microscopic measurements of the cross-sectional area of the naked axoplasmic cylinders showed that the NF population expanded radially into the solution, and electron microscopic measurements of the spacing between the NFs showed that these polymers moved apart freely from one another, dispersing into the surrounding space.

In the course of our studies on the radial mobility of NFs in isolated axoplasm, we found that addition of cationic polypeptides to the AAS restricted the dispersion of the NF population. Our interest in testing the effects of these positively charged polypeptides on NF mobility was stimulated partly by the observation that keratin filaments, which have many structural and chemical similarities with NFs, are physiologically cross-linked in vivo by a naturally occurring highly charged cationic protein called filaggrin [Dale et al., 1978, 1985; Steinert et al., 1981]. NFs, like keratin filaments, are known to have an affinity for cationic molecules [Lefebvre and Mushynski, 1988; Troncoso et al., 1990]. The cation binding sites reside predominantly within the acidic C-terminal domains of the NF polypeptides [Chin et al., 1989; Leterrier et al., 1992], which project from the filament backbone to form the NF sidearms [Hisanaga and Hirokawa, 1988; for review, see Shaw, 1991]. These observations suggested that highly charged cationic proteins might also be capable of cross-linking NFs.

In this article, we describe the results of our investigation on the effects of synthetic multivalent cationic and anionic polypeptides on the mobility and the organization of NFs in isolated axoplasm. Our studies show that cationic polypeptides of sufficient length can cross-link NFs in axoplasm into compact bundles.

MATERIALS AND METHODS

Axoplasm

Live squid (Loligo pealei) were provided by the staff of the Marine Biological Laboratory in Woods Hole, MA, where most of this work was performed. Giant axons were dissected from squid and fine-cleaned of surrounding smaller nerve fibers as described by Brown and Lasek [1990]. Two-to-three centimeter lengths of fine-cleaned axons were rinsed in about 2 ml of AAS for a few minutes and then transferred into 2 ml of fresh solution in a small transparent plastic chamber containing a 3mm-deep layer of Sylgard® silicone elastomer (Dow Corning Corporation, Midland, MI). To separate the axoplasm from the axonal plasma membrane, the axon was severed at its proximal end and the axoplasm was extruded directly into the solution by applying light pressure in a single gentle sweeping motion towards the cut end of the submerged axon. In some experiments, the axoplasm was fixed to the Sylgard® base using small stainless steel pins in order to prevent movement during the subsequent measurements. These procedures have been described in more detail elsewhere [Brown and Lasek, 1990, 1993].

Artificial Axoplasm Solution

The AAS used in these investigations (“Buffer-X”) was designed by Brady et al. [1984] to simulate the small-molecule composition of the squid giant axon, and it contained 350 mM potassium aspartate, 130 mM taurine, 70 mM betaine, 50 mM glycine, 13 mM MgCl₂, 20 mM HEPES, 10 mM EGTA, 3 mM CaCl₂, and 1 mM glucose. The pH was adjusted to 7.2 with potassium hydroxide.

Analysis of the Kinetics of NF Dispersion

Axoplasm was observed under dark-field illumination with a binocular dissecting microscope and photographed using Kodak Technical Pan film (Eastman Kodak, Rochester, NY) at a magnification of ×8. Photographs were taken as soon as possible after extrusion (the actual time interval varied from 1–4 min), and then at intervals for up to 24 h. The diameter of the dark-field image was measured on the photographic negative at a minimum of three points along the axoplasm using a reticle, and the average value was used to calculate a cross-sectional area using the equations for a cylinder. The initial cross-sectional area of the segments of axoplasm varied due to natural variation in the diameters of giant axons from different squid [Brown and Lasek, 1990]. For instance, the diameter of the axes that we used for the present studies varied between 370–540 μm. To compensate for this natural diversity, we normalized our measurements for each segment of axoplasm by expressing the temporal change in cross-sectional area as a percentage of the cross-sectional area measured at the first time point. All experiments were conducted at ambient temperature, which varied from 19–22°C.

Experiments With Polylsine and Polyglutamate

For experiments with added polypeptides, the axoplasm was extruded into AAS alone and photographed immediately. The AAS was then removed as quickly as possible with a pipette and replaced with AAS containing the test polypeptide. The axoplasm was then photographed under dark-field illumination at a number of intervals as described above. The time interval between extrusion and addition of the test solution varied from 2–4.5 min.
The polypeptides that were added to the AAS were poly-L-lysine HBr (M, 3,800), poly-L-lysine HCl (M, 36,500), and poly-L-glutamic acid (M, 36,240, sodium salt), all purchased from Sigma Chemical Co. (St. Louis, MO). The pH of all solutions was between 7.0 and 7.4. The degree of polymerization, which is the average number of amino acid residues per polypeptide chain, was 18 for M, 3,800 polylysine, 220 for M, 36,500 polylysine, and 240 for M, 36,240 polyglutamate. The average polypeptide chain lengths were 7 nm for M, 3,800 polylysine ("short-chain" polylysine), 83 nm for M, 36,500 polylysine ("long-chain" polylysine), and 91 nm for M, 36,240 polyglutamate ("long-chain" polyglutamate). These chain lengths were calculated by multiplying the average number of amino acid residues by 0.38 nm, which is the fixed distance ("virtual bond length") between the alpha-carbon atoms of the planar trans peptide repeating unit in the polypeptide backbone [Cantor and Schimmel, 1980]. Since these proteins occupy a "random coil" configuration in neutral solution (see Discussion), these chain lengths represent the average maximum end-to-end distance of the fully extended polypeptides and not necessarily the average end-to-end distance of these molecules in their solution conformation.

**Electron Microscopy**

For fixation, the AAS was removed carefully from the dish with a pipette and the axoplasm was then flooded with fixative containing 0.1 M potassium phosphate, 0.6 M sucrose, 1 mM potassium EGTA, 10 mM MgCl₂, 2.5% (v/v) glutaraldehyde, pH 7.1 [Miller and Lasek, 1985]. The fixative was changed many times in quick succession to ensure complete removal of the AAS, which contains components that react with glutaraldehyde. The fixed axoplasm was then post-fixed with osmium tetroxide, stained en bloc with uranyl acetate, dehydrated through a graded series of ethanol solutions into propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, PA). Thin sections were stained with lead citrate and uranyl acetate and examined in a Jeol 100CX electron microscope.

**RESULTS**

**Light Microscopy**

a) Axoplasm expands in artificial axoplasm solution. Figure 1A shows a cylinder of axoplasm 1 min after extrusion directly into AAS. The isolated axoplasm is almost transparent, but it can be observed using dark-field illumination because it scatters light; under dark-field illumination the axoplasm appears as a brightly illuminated image that contrasts sharply against the dark background of the surrounding homogeneous solution. Most of the light-scattering is produced by the NFs, which are the most prevalent cytological elements in squid axoplasm [Metuzals et al., 1983b; Brown and Lasek, 1990, 1993]. Previous studies have shown that such dark-field images of isolated axoplasm can be used to quantify the volume occupied by the NF population [Brown and Lasek, 1993].

Figure 1B,C shows the same axoplasmic cylinder at 100 min and 600 min after extrusion into AAS. The axoplasm retains the smooth cylindrical shape of the axon from which it was extruded but expands radially and continuously for many hours [Brown and Lasek, 1993]. For example, the diameter of the axoplasm in Figure 1 was 0.6 mm after 1 min, 0.84 mm after 100 min, and 0.94 mm after 600 min. This corresponds to an increase in cross-sectional area of 96% between 1 and 100 min and a further increase of 26% between 100 and 600 min. Overall, there was an increase in cross-sectional area of 146% between 1 and 600 min.

To further quantify the expansion of axoplasm and its NF population, we determined the cross-sectional area of the axoplasm at a series of intervals ranging from 1 to 1,300 min after extrusion into AAS. Figure 2A shows detailed kinetics for four segments of axoplasm. As we have described previously, the cross-sectional area increased rapidly during the first 100 min, and then continued to increase at a progressively slower rate for more than 1,200 min [Brown and Lasek, 1993]. Interpolation on the graph in Figure 2A indicates that the cross-sectional area had increased by 76–101% (average = 91%, n = 4) after 100 min, 109–120% (average = 113%, n = 4) after 750 min, and 115–135% (average = 125%, n = 2) after 1,300 min.

b) Long-chain polylysine inhibits axoplasmic expansion and condenses the axoplasm. Figure 2B–E shows the effects of different concentrations of long-chain (83 nm) polylysine on the radial expansion of axoplasm in AAS. In the presence of 0.001 mg/ml (0.027 μM) polylysine, the kinetics of expansion were similar to the control but the extent of expansion was less. For example, the cross-sectional area after 1,300 min had increased by 67% and 101% (average = 84%) for the two axoplasmic cylinders in 0.001 mg/ml polylysine (Fig. 2B), and by 115% and 135% (average = 125%) for the two axoplasmic cylinders in the control (Fig. 2A).

At higher concentrations of polylysine the kinetics of expansion were strikingly different from the control (Fig. 2C–E). In the presence of 0.01 mg/ml (0.27 μM) polylysine, the cross-sectional area increased rapidly for 30 min after extrusion but then began to decrease, suggesting a condensation of the cytoskeletal polymers (Fig. 2C). In the presence of 0.1 mg/ml (2.7 μM) and 1 mg/ml (27 μM) polylysine, this inhibitory effect on expansion was similar but more marked (Fig. 2D,E). For example,
the time required for expansion to cease was 30 min in 0.01 mg/ml polylysine, 10 min in 0.1 mg/ml polylysine, and less than 1 min in 1 mg/ml polylysine.

The rate and extent of condensation were also dependent on the polylysine concentration. For example, the cross-sectional area of the axoplasm in 0.01 mg/ml polylysine continued to decrease gradually for at least 1,300 min, at which time the axoplasm had condensed to slightly less than the original cross-sectional area (Fig. 2C). In the presence of 0.1 mg/ml polylysine, the condensation was more rapid and extensive, stabilizing after about 600 min at a cross-sectional area about 35% less than the original cross-sectional area (Fig. 2D). In the presence of 1 mg/ml polylysine, the condensation was even more rapid and extensive, decreasing the original axoplasmic cross-sectional area by 50% within 30 min (Fig. 2E). After 600 min the cross-sectional area had decreased to about 55% less than the original cross-sectional area, which represents more than a two-fold overall decrease in cross-sectional area.

These results show that long-chain polylysine produced a concentration-dependent inhibition of axoplasmic expansion at concentrations of 0.001 mg/ml or greater, and a concentration-dependent condensation of axoplasm at concentrations of 0.01 mg/ml or greater. This suggests that long-chain polylysine can increase the interactions between neighboring NFs in isolated axoplasm, thereby restricting the side-to-side diffusive movements that normally operate to move these polymers apart. At concentrations of 0.01 mg/ml or greater these interactions are apparently sufficiently strong to draw the NFs together into a more compact configuration, causing condensation of the axoplasm.

**c) Long chain polylysine increases the amount of light scattering.** In addition to its effects on cross-sectional area, long-chain polylysine also increased the brightness of the axoplasm under dark-field illumination. For example, Figure 3A shows a segment of axoplasm after 1 min in the presence of 0.1 mg/ml long-chain polylysine and Figure 3B shows the same axoplasm after 110 min. The increased brightness of the dark-field image in Figure 3B is indicative of an increase in the amount of light scattered by the axoplasm. No increase in light scattering was ever observed when axoplasm was allowed to expand in AAS alone (Fig. 1).

The increase in light scattering was dependent on the polylysine concentration and appeared to correlate with condensation of the axoplasm. Specifically, the increase in light scattering appeared more rapidly at higher polylysine concentrations and was only observed at concentrations that also caused a decrease in cross-sectional area. For example, there was a noticeable increase in the brightness of the dark-field image of the axoplasm within 30 min in the presence of 0.01 mg/ml polylysine, and within 1 min in the presence of 1 mg/ml polylysine. In all cases, the increased light scattering appeared at the sur-
Fig. 2. Kinetics of expansion of axoplasm in artificial axoplasm solution alone (A) and in artificial axoplasm solution containing added polypeptides (B-H). The added polypeptides were long-chain (83 nm) polylysine (B-E); short-chain (7 nm) polylysine (F), and long-chain (91 nm) polyglutamate (G,H). The percentage change in the cross-sectional area of the axoplasm (relative to the first measurement) is plotted against the time after extrusion. For each graph, each symbol-set represents the measurements for a separate piece of axoplasm. Data for four separate segments of axoplasm are plotted in each of graphs A, C and D, and data for two separate segments of axoplasm are plotted in each of graphs B, E, F, G and H.
Fig. 3. Long-chain polylysine causes an increase in the amount of light scattered by axoplasm. A: Dark-field image of a cylinder of axoplasm photographed after 1 min in artificial axoplasm solution that contained 0.1 mg/ml long-chain (83 nm) polylysine; B: same segment of axoplasm after 110 min. C: Dark-field image of a cylinder of axoplasm photographed after 1 min in artificial axoplasm solution that contained 0.1 mg/ml short-chain (7 nm) polylysine; D: same segment of axoplasm after 110 min. All four images were photographed and printed using identical exposures in order to allow direct comparison of the intensity of the light-scattering. The cross-sectional areas were 0.39 mm² in A and 0.35 mm² in B, which represents a decrease of 10%. The cross-sectional areas were 0.23 mm² in C and 0.49 mm² in D, which represents an increase of 113%. Scale bar = 0.5mm.

face of the axoplasm initially and subsequently progressed deeper into the axoplasm.

An increase in light scattering by polymers in solution is indicative of lateral aggregation [Tanford, 1961], and this suggests that the condensation of axoplasm by long-chain polylysine is accompanied by lateral aggregation of the cytoskeletal polymers. The time-dependent progression of the increased light scattering from the outer to the inner regions of the axoplasm probably corresponds to diffusion of the polylysine into the axoplasm. As the polylysine penetrates the axoplasm, it apparently interacts with the light-scattering polymers to produce polymer aggregates that scatter more light.

d) Long-chain polylysine binds tightly to elements of the axoplasm. To test whether the condensation of axoplasm in the presence of long-chain polylysine was reversible, we treated two segments of axoplasm with AAS containing 0.1 mg/ml long-chain polylysine for 20 min, and then replaced the polylysine solution with AAS lacking polylysine. Prior to removal of the polylysine, the cross-sectional areas had begun to decrease (see Fig. 1D) and the light-scattering of the axoplasm had increased noticeably. After 1,200 min in AAS lacking polylysine, the intensity of the light-scattering had not diminished and the cross-sectional areas of the two axoplasmic cylinders had increased by only 2 and 8% of the cross-sectional area immediately prior to removal of the polylysine. The persistence of the elevated light-scattering and the small extent of axoplasmic expansion after removal of the polylysine indicates that the condensation of axoplasm was not freely reversible and this suggests that the long-chain polylysine molecules bound tightly to the axoplasmic polymers.

e) Short-chain polylysine does not inhibit expansion. To test whether the inhibition of axoplasmic expansion by polylysine was dependent on polypeptide length, we examined the effects of a smaller polylysine molecule (Mₐ 3,800) with a chain length of 7 nM. In the presence of 0.1 mg/ml (26 µM) of this short-chain polylysine the cross-sectional area increased rapidly during the first 100 min, and then continued to increase at a progressively slower rate for more than 1,000 min (Fig. 2F). After 1,100 min, the cross-sectional areas of the two cylinders of axoplasm in 0.1 mg/ml short-chain polylysine had increased by 128 and 146%. These kinetics are similar to those of the control, in which axoplasm was allowed to expand in AAS alone (Fig. 2A). Thus, in striking contrast to long-chain polylysine (Fig. 2D), comparable concentrations of short-chain polylysine did not inhibit the expansion of axoplasm.

Short-chain polylysine also had no effect on the amount of light scattered by the axoplasm. For example, Figure 3C shows a segment of axoplasm after 1 min in the presence of 0.1 mg/ml short-chain polylysine and Figure 3D shows the same axoplasm after 110 min. As for the control (Fig. 1), the axoplasm expanded greatly between 1 and 110 min and the brightness of the dark-field image changed very little. By contrast, 0.1 mg/ml long-chain polylysine inhibited expansion and greatly increased the brightness of the axoplasm (Fig. 3A,B). These observations suggest that, in contrast to long-chain polylysine, short-chain polylysine did not induce lateral aggregation of the cytoskeletal polymers.
We have also studied the effects of a much smaller polycation, spermidine (M, 145), on the expansion of axoplasm in AAS. Spermidine concentrations as high as 10 mg/ml neither inhibited axoplasmic expansion nor increased the intensity of the light scattering (data not shown). The fact that short-chain polylysine and spermidine had no effect on the expansion or light scattering of axoplasm supports the inference that the inhibition of expansion by long-chain polylysine was due to intermolecular cross-linking of the cytoskeletal polymers and required a molecule that was sufficiently long to bridge the distance between neighboring polymers in the axoplasm.

f) Long-chain polyglutamate is 100-fold less effective than long-chain polylysine. To test whether the inhibition of axoplasmic expansion by long-chain polylysine was dependent on the charge of this protein, we investigated the expansion of axoplasm in the presence of long-chain polyglutamate (M, 36,500), a multivalent anionic polypeptide with a chain length of 91 nm. This polypeptide is similar in size to the long-chain polylysine that we used, but opposite in charge. Figure 2G shows the change in cross-sectional area for two cylinders of axoplasm in the presence of 0.1 mg/ml long-chain polyglutamate. The kinetics of expansion were similar to the control (Fig. 2A) but the extent of expansion was less. For example, the cross-sectional area after 1,300 min had increased by 90 and 105% (average = 98%) for the two axoplasmic cylinders in 0.1 mg/ml polyglutamate (Fig. 2G), and by 115 and 135% (average = 125%) for the two axoplasmic cylinders in the control (Fig. 2A). In the presence of 0.5 mg/ml polyglutamate, the extent of expansion was reduced further (Fig. 2H). For example, the cross-sectional area after 950 min had increased by 58 and 69% (average 64%) for the two axoplasmic cylinders in 0.5 mg/ml polyglutamate (Fig. 2H), and by 111 and 113% (average = 112%) for the two axoplasmic cylinders in the control (Fig. 2A). Visual inspection of the dark-field micrographs of control axoplasm after 1 min and 100 min in AAS. As we have described elsewhere [Brown and Lasek, 1993], the NFs in expanded axoplasm (Fig. 4B) were organized more loosely and were spaced further apart than in axoplasm after only 1 min in AAS (Fig. 4A). The wider spacing between the NFs is consistent with the inference that these polymers are only weakly interactive, moving apart freely in response to the forces of Brownian motion that continually bombard them [Price et al., 1988; Brown and Lasek, 1993].

b) Axoplasm condensed with 0.1 mg/ml long-chain polylysine. Figure 5 shows the ultrastructure of axoplasm after 100 min in 0.1 mg/ml long-chain polylysine. By this time, our measurements indicate that the axoplasm had condensed back to approximately the cross-sectional area that it had initially, though the condensation had not progressed to completion (Fig. 2D). We compared the peripheral and central regions of the axoplasm because our observations on the light scattering suggested that NF bundling occurred peripherally at first, and then subsequently more centrally (see above). Within the outer 20 μm of the axoplasm, which represented about one half of the total cross-sectional area, most NFs were clustered tightly together in bundles and very few isolated NFs were observed (Fig. 5A). More centrally, few NFs were bundled and many isolated NFs were observed (Fig. 5B). In contrast to the appearance of axoplasm after 100 min in 0.1 mg/ml long-chain polylysine, no NF bundling was observed in electron micrographs of axoplasm after 100 min in 0.1 mg/ml short-chain polylysine or 0.1 mg/ml long-chain polyglutamate (data not shown), and this is consistent with our observation that the light scattering of the axoplasm did not increase in the presence of these proteins (for example, see Fig. 3C,D).
Figure 6A shows an electron micrograph of the NF bundles at higher magnification after 100 min in 0.1 mg/ml long-chain polylysine. The NFs were invested by a densely staining material that obscured the polymers within the core of the bundle, but individual NFs could be identified clearly at the edges of these aggregates. The polymers within the bundles were aligned in parallel so that all of the polymers in a given bundle were sectioned in the same orientation. In addition to the bundles that contained only NFs, we also observed some bundles that contained MTs as well as NFs, and others that contained only MTs (Fig. 6B). These MT-containing bundles were most numerous at the extreme periphery of the axoplasm (within a few μm of the surface), where many bundles of three or more MTs were observed. This is consistent with electron microscopic observations of axoplasm in the intact squid giant axon which have shown that MTs are most numerous in the peripheral axoplasm that underlies the plasma membrane [Metuzals et al., 1983a; Sakai et al., 1985].

These ultrastructural observations indicate that long-chain polylysine binds tightly to neighboring NFs in isolated axoplasm and cross-links them together into tightly packed bundles, thereby preventing the normal tendency for these polymers to disperse radially into the surrounding solution.

DISCUSSION

We have studied the effects of synthetic cationic and anionic polypeptides on the dispersion of NFs in isolated axoplasm immersed in AAS. At concentrations of 0.001 mg/ml or greater, long-chain (83 nm) polylysine inhibited NF dispersion. At concentrations of 0.01 mg/ml or greater, the polylysine bundled the NFs together and condensed the cross-sectional area of the axoplasm. Inspection of the NF bundles by electron microscopy revealed that the NFs were closely spaced, aligned in parallel, and invested by an electron dense material. Apparently, the 83 nm polylysine can bind tightly to NFs in axoplasm, thereby increasing their interactions and cross-linking them together.

The Mechanism of NF Bundling by Polylysine

In the presence of 1 mg/ml (27 μM) long-chain polylysine, the axoplasm condensed to less than half of its original cross-sectional area. This indicates that axoplasm contains a large excess of free space within which the NFs can move by the ordinary forces of thermal motion. In the axon, this excess space permits the NFs to express their inherent tendency to move from side-to-side and distribute throughout the axonal cross-sectional area in response to the randomizing forces of Brownian
Fig. 5. Electron micrographs of axoplasm in transverse section after 100 min in artificial axoplasm solution that contained 0.1 mg/ml long-chain (83 nm) polylysine. The micrograph in (A) was taken towards the periphery of the axoplasm and the micrograph in (B) was taken towards the center of the axoplasm. Scale bar = 0.5 μm.
motion [Price et al., 1988, 1990; Brown and Lasek, 1993]. We propose that NFs tend to make transient contacts with their neighbors during their stochastic side-to-side movements, and that polylysine acts to stabilize these interactions. In this way, polylysine reveals the numerous collisions that continually occur between these polymers in axoplasm.

In healthy axons, the interactions between NFs are weak and thus the polymers remain free to move apart after they happen to collide with one another. By cross-linking the NFs, polylysine holds these polymers together so that their movements become constrained. Apparently, this cross-linking process together with the continual motion of the NFs further arranges these polymers side-by-side into tightly packed bundles in which the NFs are linked at so many points along their lengths that the ordinary thermal forces of Brownian motion are no longer sufficient to move them independently of their neighbors.

This bundling effect of polylysine differs from the effects of another cross-linker, glutaraldehyde. With glutaraldehyde, axoplasm is also extensively cross-linked and the mobility of the NFs is also constrained. However, glutaraldehyde does not cause the NFs to form bundles. In addition, glutaraldehyde causes less condensation of axoplasm. For example, the axoplasmic cross-sectional area decreased by 9–24% (average = 15%, n = 4) over a period of 1,200 min in the presence of 1% (0.1 M) glutaraldehyde in an isosmotic phosphate buffer (data not shown), and by more than 50% in the presence of 1 mg/ml (27 μM) long-chain polylysine in AAS (Fig. 2E). This suggests that the cross-links produced by glutaraldehyde are more able to withstand the ordinary thermal forces of Brownian motion than those produced by polylysine. These differences can be accounted for by the size and cross-linking mechanisms of these two agents; glutaraldehyde is a small molecule that forms covalent cross-links between all proteins, regardless of their charge, whereas polylysine is a large flexible molecule that forms non-covalent electrostatic interactions only with proteins that bear a negative charge. Apparently, the covalent bonds formed by glutaraldehyde stabilize a sufficient number of interactions within and between the continually moving NFs to preserve their scattered distribution in the radial dimension. In this way, glutaraldehyde cross-linking reveals a "snapshot" of the stochastic effects of Brownian motion on these polymers [see Price et al., 1988 for further discussion].

### Polysine Chain Length and NF Cross-Linking

The cross-linking of NFs by polysine was dependent on the length of the polylysine molecules. Specifically, 0.1 mg/ml of the short-chain polylysine (polypep-
tide chain length = 7 nm) did not induce bundling of the NFs and did not inhibit their dispersion, whereas an equal weight concentration of the long-chain polylysine (polypeptide chain length = 83 nm) caused extensive cross-linking and led to condensation of the axoplasm. Notably, the maximum end-to-end distance of this long-chain polylysine is similar to the average nearest-neighbor distance between NFs in squid axoplasm, which we found to be 81 nm [Brown and Lasek, 1993]. Apparently, the short-chain polylysine molecules were not sufficiently long to effectively stabilize the transient contacts that continually occur between adjacent NFs in axoplasm.

Polysine Cross-Linking and NF Sidearms

Physical studies on polysine and polyglutamate have shown that these polypeptides both adopt a “random coil” conformation in aqueous solution at neutral pH [Doty, 1959]. In the present study, we compared the effects of a long-chain polylysine molecule (chain length = 83 nm) with a long-chain polyglutamate molecule of similar length (chain length = 91 nm). Because these two polypeptides have a similar chain length and adopt a similar conformation, they should have a similar size and shape in AAS. However, our results indicate that the long-chain polylysine cross-linked the NFs into bundles whereas equivalent concentrations of the long-chain polyglutamate did not. Long-chain polyglutamate was found to partially inhibit the radial expansion of axoplasm, but the concentration required to achieve this was 100-fold higher than for long-chain polylysine. This partial inhibition of expansion by higher concentrations of long-chain polyglutamate may not necessarily be due to cross-linking, but this could be determined by examining even higher concentrations of polyglutamate to determine whether bundling of NFs occurs. Nevertheless, these results indicate that the cross-linking of NFs by polylysine is charge-dependent. Apparently, NFs have a much higher affinity for polylysine, which is basic, than for polyglutamate, which is acidic.

NFs have also been found to aggregate into bundles in vitro in the presence of cytochrome-c [Gilbert et al., 1975; Gilbert, 1976], which is a naturally occurring basic protein with a pI of 10.6 [Lehnninger, 1975], and Chin et al. [1989] have shown that this protein binds to NFs in a saturable manner. In addition, NFs are known to bind inorganic cations such as Ca$^{2+}$ and Al$^{3+}$ [Lefebvre and Mushynski, 1988] and these cations can also induce NFs to aggregate under certain conditions in vitro [Troncoso et al., 1990; Leterrier et al., 1992].

The affinity of NFs for cationic molecules and ions is due principally to their highly charged side-sidearms which are composed of the carboxy-terminal tails of the NF proteins [Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988]. These carboxy-terminal domains have an abundance and predominance of acidic amino acid residues and they are also extensively phosphorylated in axons [for review see Shaw, 1991]. The predominance of acidic residues in the carboxy-terminal domains makes the overall charge balance of the NF sidearms highly acidic and this explains the affinity of NFs for positively charged molecules such as polylysine.

The charge-dependent cross-linking of NFs by exogenous basic proteins has a physiological counterpart in the cross-linking of keratin filaments by a naturally occurring basic protein called filaggrin [Dale et al., 1978, 1985; Steinert et al., 1981]. Like NFs, keratin filaments are members of the evolutionarily related intermediate filament family. During the development of the stratum corneum of the epidermis, keratin filaments are cross-linked by filaggrin into dense bundles called macrofibrils. The filaggrins in their cross-linking state are very basic proteins with a pI > 10, and they bind tightly to the acidic carboxy-terminal domains of the keratin subunits, thereby condensing adjacent keratin filaments into tight bundles. In this way, the filaggrins constrain the mobility of the keratin filaments producing an extremely stable cytoskeletal architecture, which contributes to the structural durability of the integument.

Neurofilaments Are Normally Spaced Apart From Each Other in Axons

The bundling of NFs in the presence of polysine is particularly striking because it differs so markedly from the normal distribution of these cytoskeletal polymers in axons. Normally, axonal NFs are spaced apart from each other and show no tendency to aggregate together. Even in the most densely packed regions of axons, NFs are always separated from their neighbors [Price et al., 1990, 1993; Reles and Friede, 1991].

Our studies on the interaction of NFs with charged proteins suggest that the normal tendency of these cytoskeletal polymers to remain apart in axons could be due to their unusual charge properties. Specifically, the observation that polyglutamate is a relatively poor cross-linker of NFs implies that these polymers have a relatively low affinity for acidic proteins. Thus, the highly acidic nature of NF sidearms may normally serve to minimize the potential for interaction between neighboring NFs, thereby ensuring the independence and lateral mobility of these polymers in the radial dimension of the axon.

The proposal that neurofilament sidearms have a weak affinity for each other and for neurofilaments as a whole is supported by the studies of Chin et al. [1989]. These authors used limited proteolysis to isolate the carboxy-terminal portions of the NF-M and NF-H polypeptides from native bovine neurofilaments and then puri-
Neurofilament Bundling in Giant Axonal Neuropathy

In contrast to healthy axons, tight bundles of NFs have been described in certain neuropathological conditions, such as in human inherited giant axonal neuropathy [Donachy et al., 1988]. In giant axonal neuropathy, these tight bundles are surrounded by large numbers of other densely packed NFs which distend the axons producing the giant axonal balloons for which this condition is named. The parallel arrangement and tight packing of the bundled NFs indicates that these polymers are held together by bonds which are stronger than the thermal forces of Brownian motion that are usually sufficient to separate these polymers in axons [Price et al., 1988, 1990, 1993].

Donachy et al. [1988] proposed that giant axonal neuropathy may be caused by an increase in the strength of association between axonal NFs. Our observations on the charge-dependent cross-linking of NFs by polylysine suggest that such abnormal associations could be due to modification of the charge properties of the NFs or of proteins that associate with them. These abnormal interactions could contribute to the abnormal accumulations of NFs that distend axons. Specifically, by constraining the normally free and independent lateral mobility of NFs, such abnormal associations could also interfere with the longitudinal movements of these polymers, which are transported down the axon by the mechanisms of slow axonal transport.

ACKNOWLEDGMENTS

We are especially grateful to Dr. Robert H. Miller for his generous help and advice and we also thank Diane Kofskey for her technical assistance. This research was supported by a grant from the National Institutes of Health to R.J. Lasek.

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