Experimental Increase of Neurofilament Transport Rate: Decreases in Neurofilament Number and in Axon Diameter

SALVATORE MONACO, M.D., LUCILA AUTILIO-GAMBETTI, PH.D.,
RAYMOND J. LAECK, PH.D., MICHAEL J. KATZ, PH.D.,
AND PIERLUIGI GAMBETTI, M.D.

Abstract. In 2,5-hexanediene (2,5-HD)-induced axonal neuropathy, the rate of neurofilament (NF) transport increases in optic axons. To test the prediction that increases in the rate of polymer transport in any one locality of the axon lead directly to a decrease in the number of NF in that locality, NF and microtubules (MT) were quantitatively analyzed in axonal cross sections. In 2,5-HD axons the number of NF was 38% of that in control axons while the number of MT was not significantly changed; it appears that the drug treatment decreases NF number in the proximal axon regions, most directly through an increase in rate of NF transport. In those regions, the cross-sectional areas of the 2,5-HD-treated axons were 45% smaller than those of control axons; although the axons had shrunken in diameter, they retained their normal cylindrical shapes as measured by the index of circularity. Reduced internal expansive forces in the axon, working in conjunction with the normal external compressive forces, appear to reduce the radius of the axon. Quantitative analyses demonstrated that the average and the maximum lateral spacings between NF-NF, NF-MT, and MT-MT were all 30% larger in 2,5-HD-treated axons than in control axons. This suggests that polymers are relatively free to move laterally away from one another and to fill the available space within the axon. These observations are not consistent with models wherein 2,5-HD acts to crosslink the NF into an immobile network that can no longer advance within the axon. Instead, it appears more likely that 2,5-HD acts selectively on the interaction between some NF and the slow transport mechanism to increase the rate of NF transport.

Key Words: Axonal caliber; Axonal transport; Microtubules; Morphometry; Nearest-neighbor distance; Neurofilaments; 2,5-Hexanediene.

INTRODUCTION

The radial dimension of an axon directly correlates with the number of polymers and other organelles contained within the axon. In myelinated axons, neurofilaments (NF) and microtubules (MT) are the most numerous structures. The proteins that constitute these structures are synthesized in the cell body and are then conveyed through the axon by slow axonal transport (1). According to the "structural hypothesis" of axonal transport, polymers are assembled in the cell body and are then transported as cytological structures through the axon by a polymer sliding mechanism (2–4).

One prediction of this model is that the rate of polymer transport through any segment of the axon will directly affect the number of polymers that are present at any one time in that segment (5, 6). For example, if the number of polymers exported from the nerve cell body remains constant and the rate of polymer transport through a region of the axon increases, then the number of polymers in that region should

From the Division of Neuropathology, Institute of Pathology (SM, LA-G, PG) and the Bio-architectonics Center (RJL, MJK), School of Medicine, Case Western Reserve University, Cleveland, Ohio. Correspondence to: Pierluigi Gambetti, M.D., Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44106. Supported by NIH Grants NS 14509 and AG 00795.
decrease. The recent finding that certain neurotoxicological agents selectively increase the rate of slow axonal transport provides the opportunity to test this prediction (7–9). One of these agents, 2,5-hexanediol (2,5-HD), has attracted considerable interest because it provides an experimental model for the axonal changes characteristic of the acquired and familial forms of human giant axonal neuropathies (10). Chronic administration of 2,5-HD causes the formation of NF-filled enlargements in the distal regions of central and peripheral axons and increases the rate of transport of NF proteins in the segment of the optic and sciatic axons proximal to the enlargements (7, 11). In normal optic axons, NF are transported with slow component a at 0.25 mm/day, but in 2,5-HD axons, a significant fraction of the NF are transported at rates comparable to the more rapidly moving slow component b (SCb) wave at 2 mm/day (12, Monaco et al, unpublished data).

If the amount of synthesis and export of NF from the nerve cell body into the axon remains constant in 2,5-HD-treated animals, then the number of NF found at any one locale within the axon should decrease. To test experimentally such theoretical predictions in optic axons, we compared the cross-sectional areas and the numbers of NF and MT in optic axons of 2,5-HD and control axons. In addition, the interactions between the polymers were quantitatively compared by determining the nearest-neighbor relationships of NF and MT.

MATERIALS AND METHODS

Six male Sprague-Dawley rats, 12 weeks (wk) old at the beginning of the experiment, were used for the present study. Three rats were given 0.5% 2,5-HD in drinking water continuously for eleven wk; three animals, used as controls, received regular tap water.

Experimental and control rats were perfused at eleven wk of treatment (23-wk-old), as in previous studies (7). The primary optic pathway (optic nerve, optic tract, and superior colliculus) was sampled at 2 mm intervals. All specimens were embedded in Spurr as previously described (7).

For the analysis of axonal cross-sectional area and index of circularity, electron micrographs (EM) (×2,700) of systematically sampled areas (13) from the optic nerves, 5 mm from the eyeball were taken with a JEOL 100 CX electron microscope. A calibration grid was photographed at the beginning and at the end of every EM session; the focus setting was not changed during the session. Pictures were printed at a final magnification of ×10,000. The area of individual axon was determined by a computer-assisted digitizing system (BIOQUANT). We measured a total of 6,584 axons from control animals (1,908, 1,145, and 3,631 from control animals 1, 2 and 3) and of 10,182 axons from 2,5-HD-treated animals (2,526, 1,909 and 5,747 from 2,5-HD animals 1, 2 and 3).

For quantitative analysis of MT and NF, a subset of the same optic axons was rephotographed at ×10,000 and printed to a final magnification of ×30,000. Only axons with MT and NF oriented in cross-section were used. Determination of the number of MT and NF was obtained by direct count in a total of 214 control axons (43, 107 and 54 axons from control 1, 2 and 3) and of 273 experimental axons (84, 65 and 120 axons from 2,5-HD animals 1, 2 and 3) ranging from 0.1 to 2 μm² in cross-sectional area, with a size distribution representative of that of the entire axonal population. The area occupied by mitochondria and smooth endoplasmic reticulum was subtracted from the total area of the axon in both control and experimental animals. The number of MT and NF were plotted as a function of axonal cross-sectional area.

The length of clearly identifiable sidearms connecting NF to NF, MT to MT, and MT to NF was measured by using a calibrated eyepiece. Where no sidearms were observed, the minimum distance between these individual structures was measured; 112 nm was taken as the cut-off point because this was the maximum length found for viable sidearms in experimental axons. Individual distances were taken from the center of a given MT or NF to the center of the nearest MT or NF (14). A total of 27 axons from controls and 27 axons from
INCREASE OF NEUROFILAMENT TRANSPORT RATE

Fig. 1. Percent distribution of optic axons as a function of cross-sectional area. Both control (open bars) and 2,5-HD axons (solid bars) have a unimodal distribution. All values for the 2,5-HD axons are displaced toward the left indicating axons of all sizes are decreased in size following administration of 2,5-HD (p < 2 × 10⁻⁵, Kolmogorov-Smirnov two-group test). Based on the examination of 6,684 control and 10,182 2,5-HD axons from three control and three experimental animals.

Experimental animals, randomly selected, were examined on pictures at a final magnification of ×80,000. The circularity factor for axons was calculated as described by Dyck et al (13). Statistical analyses included regression, Student's t-test and Kolmogorov-Smirnov two-group test.

RESULTS

Clinical manifestations and pathologic changes of optic axons were found to be as previously reported (7). Briefly, rats exposed to 2,5-HD showed a progressive hindlimb weakness by the fifth to the sixth wk of treatment; this change was followed by body weight loss and by weakness of the forelimbs, with muscular atrophy.

Axonal enlargements with accumulation of NF, that are first detected in the stratum opticum of the superior colliculus at the fifth to the sixth wk of intoxication (7) had extended proximally to the distal two thirds of the optic tract. As in our previous study, there was no evidence of axonal degeneration.

Cross-Sectional Area: Axons in the optic nerve of 2,5-HD-treated rats, 5 mm from the sclera, were smaller than those of control animals. Figure 1 illustrates the distribution of cross-sectional areas of optic axons from the three control and the three 2,5-HD-treated animals. Comparison of the shapes of these curves indicates that treatment with 2,5-HD shifted the entire population of optic axons toward smaller diameters. The mean cross-sectional area of the axons from the 2,5-HD-treated rats (0.489 ± 0.07 µm², n = 3) was 45.5 ± 6.0% smaller than that of control axons (0.896 ± 0.07 µm², n = 3). This difference was statistically significant (Student t-test, p < 0.003). In control animals, 57% of the axons were between 0.1 to 0.7 µm² with a mode at 0.4 µm². In 2,5-HD animals, only 7% of the axons were larger than 1 µm² and 53% of the axons ranged between 0.1 and 0.4 µm² with a mode of 0.25 ± 0.01 µm².

Although 2,5-HD treatment reduced axon size, the axons retained their normal cylindrical shape. The extent to which the shape of an axon departs from that of a perfect cylinder is measured with the index of circularity, which is defined as the ratio of the cross-sectional area of an axon to the area of a circle having the same
circumference (15). Normal peripheral axons have been shown to have a shape similar to that of a true cylinder and an index of circularity close to 1. When peripheral axons undergo atrophy, the index of circularity decreases significantly. In our study, the index of circularity for 2,5-HD axons (0.829 ± 0.11) did not differ from that of control axons (0.802 ± 0.11). This indicates that 2,5-HD treatment produces axonal atrophy in optic axons without a change in axonal shape.

**Neurofilament and Microtubule Number:** Visual inspection of electron micrographs suggested that the control axons had many more NF than did 2,5-HD axons of comparable size (Fig. 2). After counting all the NF and MT in randomly sampled axons from the optic nerve of three 2,5-HD-treated and three control animals we found that the mean number of NF in 2,5-HD axons was 16.8 ± 1.5 (n = 3) while in control axons of similar size it was 47.2 ± 8.7 (n = 3). This difference was significant (p < 0.004) and corresponds to a 64.3 ± 3.2% decrease in the mean number of NF in 2,5-HD axons as compared to controls. In contrast, the mean number of MT in 2,5-HD axons (28.8 ± 0.3) was not significantly different from that of controls (30.1 ± 1.3).

Histograms of the number of NF plotted in relation to axon size showed that number of NF increased with axon size in both the control and the 2,5-HD axons (Fig. 3A). The number of NF was decreased in 2,5-HD axons of all sizes and on average was 38.1% ± 6.0 that of control axons of similar size (p < 0.0001).

The number of MT also increased with axonal size in both the control and the 2,5-HD axons (Fig. 3B) and in all the different size groups of axons appeared to maintain the usual correlation with axonal volume.

**Neurofilament and Microtubule Spacings:** Visual inspection of the electron micrographs suggested that the cytoskeletal packing density decreased in the 2,5-HD axons; in other words, it appeared that the spacing between polymers increased in experimental axons. To verify this possibility, we quantified the spacing of the polymers in axons by measuring the center-to-center distance between the polymers. With two kinds of polymers, NF and MT, there are three possible combinations of neighbor relations: NF-NF, NF-MT, and MT-MT. Figure 4 and Table 1 compare the distributions of these nearest-neighbor relations in control and in 2,5-HD axons.

The nearest-neighbor distance between NF-NF in control axons had a mean of 40.6 nm and a mode at 40 nm, values similar to those previously reported (16, 17). In 2,5-HD axons the mean distance was 53.2 and the mode 48 nm. In both control and 2,5-HD axons, none of the distances were lower than 24 nm (Fig. 4A; Table 1).

Like the NF, the MT were more widely spaced in the 2,5-HD axons than in the control axons (Fig. 4B; Table 1). In control axons, the nearest-neighbor distances between MT-MT had a mean of 54.7 nm and a mode at 48 nm while in 2,5-HD axons, the mean was 70.6 and the mode at 72 nm. The nearest-neighbor spacing between NF-MT was also larger in the 2,5-HD axons than in the control axons (Fig. 4C).

Thus, comparison of the means of the nearest-neighbor spacings in the 2,5-HD and control axons indicates that the spacing of the NF-NF, NF-MT, and MT-MT were all approximately 30% greater than in controls.

**DISCUSSION**

*Transport Rate Is a Determinant of Local Polymer Number in Axons:* In 2,5-HD-treated animals, we found that the number of NF present in cross-sections of optic axons is reduced to approximately one-fourth of that in control animals. The number of NF polymers present at any one locality in an axon is determined by: a) the
Fig. 2. Electron micrograph of myelinated optic axons from control (A) and 2,5-HD-treated (B) rats. A. Although occasionally intermingled, more often microtubules (MT) and neurofilaments (NF) form microdomains. Side-arms connecting NF to NF are especially numerous and straight. Side-arms connecting MT to MT and MT to NF are less distinct, more irregular and less frequent. B. Markedly fewer NF with fuzzier and occasionally much longer side-arms (arrow) are seen in the 2,5-HD axon. The MT are evenly distributed and do not form distinct microdomains as in the control axon. Fuzzy side-arms emanate from MT. ×95,000.
Fig. 3. Histograms of the numbers of NF (A) and MT (B) plotted in relation to axon size. 2,5-HD: solid bars; control: open bars. * Group comprising axons of 1.1 to 1.5 μm². Based on analysis of 214 axons from three controls and 273 axons from three 2,5-HD-treated animals.

amount of NF protein that is synthesized, assembled, and transported from the nerve cell body into the axon, b) the rate of NF transport through the axon, and c) the rate of removal of NF from the axon (by disassembly or proteolysis) (6). Studies of axonal transport in 2,5-HD-treated animals demonstrate that NF move more rapidly in 2,5-HD-treated animals than in control animals (7). If the amount of NF inserted in the axon for transport and the rate of NF protein removal are not altered, can the transport rate changes account for the observed changes in polymer number?

From our quantitation of polymer numbers we can estimate the local kinetics of NF transport rates. At any locale, the transport rate and the NF number are related by: NoVo = NnVn (where: No is the original number of polymers in an axonal segment, Vo is the original speed, and Nn is the new number of polymers moving at a new speed Vn). Pulse-labeling studies of normal rat optic axons show that the peak transport rate of the NF is 0.2 mm/day (12). Using this rate, we estimate that in order to have the observed 75% reduction in the number of NF/axon, NF must be transported at an average rate of 0.8 mm/day in 2,5-HD axons. This rate is four times higher than normal and is within the average rate expected from our previous pulse-labeling studies (7), which showed that part of the NF are transported at a normal rate while another moves at a rate similar to that of SCb.

The number of MT per axon of the same size was not different in 2,5-HD and
TABLE 1
Nearest-Neighbor Distances Separating Neurofilaments (NF) and Microtubules (MT) in Control and 2,5-HD-Treated Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2,5-HD</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-NF</td>
<td>Mean 40.61 ± 9.3</td>
<td>53.2 ± 14.3*</td>
<td>+31</td>
</tr>
<tr>
<td></td>
<td>Mode 40</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>MT-MT</td>
<td>Mean 49.6 ± 10.9</td>
<td>64.7 ± 16.8*</td>
<td>+30</td>
</tr>
<tr>
<td></td>
<td>Mode 48</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>NF-MT</td>
<td>Mean 54.7 ± 12.1</td>
<td>70.6 ± 18.0*</td>
<td>+39</td>
</tr>
<tr>
<td></td>
<td>Mode 48</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

* Distances were measured from center-to-center of NF and MT and are expressed in nm ± SD. NF-NF: n = 640 (control) and n = 189 (2,5-HD). MT-MT: n = 354 (control) and n = 327 (2,5-HD). NF-MT: n = 252 (control) and n = 138 (2,5-HD). * p < 0.001.

control animals. However, if corrections are made for the 45% reduction in size of the 2,5-HD axons, a 28% reduction in the number of MT is found. Theoretically, such a decrease could be accounted for by an increase in the rate of MT transport; however, comparisons of the transport profiles did not show any differences in the rate of MT transport in the treated and the untreated optic axons (7, and Monaco et al, unpublished data). Thus, unlike the effects on NF, the decreased number of MT is probably due more directly to 2,5-HD effects on factors other than the rate of transport.

Local Polymer Number and the Radial Dimensions of Axonal Segments: In general, larger axons have more cytoskeletal polymers (18). This observation has led to the proposal that the local number of NF and MT will vary in direct proportion to the axonal radius at that locality. This proposal is supported by quantitative studies of slow axonal transport, NF number, and axonal radius (6, 19). For instance, if either a motor or a sensory neuron is disconnected from its target cell, then the amount of NF protein synthesis in the cell body decreases (19). This alteration produces a decrease in the amount of slowly transported NF protein in the axon, and there is a concomitant decrease in both the number of NF and the radius of the axon (19, 20). Likewise, during normal development the three-to-eight-fold decrease in rate of transport of NF and MT (21, 22) correlates with the increase in axonal radius. Our results with 2,5-HD axons also support this relationship. Specifically, we have used an experimental model to demonstrate how the rate of transport can determine the number of NF in the axon and how in this way the transport rate may influence the axonal radius. However, in 2,5-HD axons, the reduction in number of NF was more pronounced than expected for the reduction in caliber: 2,5-HD axons have a smaller number of NF than control axons of similar size. At the same time, the decrease in MT number was as expected for the corresponding reduction in axonal caliber.

Cytoskeletal Polymers Remain Loosely Associated in 2,5-HD Axons: Our analyses of the nearest-neighbor relationships between polymers in 2,5-HD and control axons show that the minimum spacing between the polymers was unaffected by 2,5-HD. The minimum spacing between polymers is the effective volume occupied by the polymers and their projecting sidearms, and our results indicate that 2,5-HD does not affect these inherent structural characteristics of the polymers. By contrast with the lack of an effect on the minimum spacing, 2,5-HD did produce an increase in both the mean and the maximum inter-polymer spacings.

These increases in inter-polymer spacings represent a "loosening" of the polymer
Fig. 4. Percent distribution of the nearest-neighbor distances between NF-NF (A), MT-MT (B) and NF-MT (C) plotted as a function of their lengths. Control: open bars; 2,5-HD-treated rats: solid bars. Note the displacement toward greater lengths of the distances separating all these structures in 2,5-HD axons. The distribution, however, remains unimodal indicating that all distances are affected in a similar way. Kolmogorov-Smirnov two-group test showed that all three frequency distributions of nearest-neighbor distances in 2,5-HD axons was significantly different from controls (p < 0.0001). Based on 27 control and 24 2,5-HD axons from three control and three experimental animals.
INCREASE OF NEUROFILAMENT TRANSPORT RATE

packing. This loosening suggests that the cytoskeletal polymers are relatively free to move laterally away from one another and to fill the available space within the axon. The polymer loosening is not consistent with models wherein 2,5-HD acts to interconnect the NF into an immobile network that can no longer advance within the axon (23–26). Instead, it appears more likely that 2,5-HD acts selectively on the interaction between NF and the mechanism of slow transport and this results in an increase of the rate of NF transport.

Conclusion: A Mechanism for Proximal Axonal Atrophy in 2,5-HD Neuropathy: Based on these observations we propose the following model for the axonal atrophy that is found in 2,5-HD-induced neuropathy: 2,5-HD increases the overall rate of NF transport without affecting the synthesis or the supply of NF from the nerve cell body and without strengthening interactions between NF. The change in the average rate of NF transport then leads directly to a decrease in the number of NF present in any one locality within the axon. Concurrently, reduced internal expansive forces, perhaps working in conjunction with the normal external compressive forces, such as those exercised by the myelin sheath, act locally to reduce the radius of the axon (27).

REFERENCES


(Received 3 August 1987/ Accepted 1 March 1988)
MS87-74