Delivery of Newly Synthesized Tubulin to Rapidly Growing Distal Axons of Rat Sympathetic Neurons in Compartmented Cultures

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Abstract. Growing axons receive a substantial supply of tubulin and other proteins delivered from sites of synthesis in the cell body by slow axonal transport. To investigate the mechanism of tubulin transport most previous studies have used in vitro models in which the transport of microtubules can be visualized during brief periods of growth. To investigate total tubulin transport in neurons displaying substantial growth over longer periods, we used rat sympathetic neurons in compartmented cultures. Tubulin synthesized during pulses of [^{35}S]methionine was separated from other proteins by immunoprecipitation with monoclonal antibodies to α and β tubulin, further separated on SDS-PAGE, and quantified by phosphorimaging. Results showed that 90% of newly synthesized tubulin moved into the distal axons within 2 d. Furthermore, the leading edge of tubulin was transported at a velocity faster than 4 mm/d, more than four times the rate of axon elongation. This velocity did not diminish with distance from the cell body, suggesting that the transport system is capable of distributing newly synthesized tubulin to growth cones throughout the axonal tree. Neither diffusion nor the en mass transport of axonal microtubules can account for the velocity and magnitude of tubulin transport that was observed. Thus, it is likely that most of the newly synthesized tubulin was supplied to the growing axonal tree in subunit form such as a heterodimer or an oligomer considerably smaller than a microtubule.

THE protein constituents of the axonal cytoskeleton are synthesized in the cell body, and much research has focused on how tubulin, the most abundant axonal protein which composes the microtubules, is transported into growing axons. It is well established that the fast-transported membrane proteins and secretory proteins are transported along axonal microtubules within vesicles powered by kinesin superfamily proteins (Vallee and Bloom, 1991; Aizawa et al., 1992). Tubulin moves along the axon via slow axonal transport (James and Austin, 1970; Karlsson and Sjöstrand, 1971; McEwen et al., 1971; Hoffman and Lasek, 1975), and neither the substrate, vehicle, nor the motor responsible for the transport of tubulin and of other slow-transported proteins has been characterized. It has been widely hypothesized that tubulin is polymerized in the cell body and that microtubules are the vehicles for tubulin transport (Black and Lasek, 1980; Lasek, 1980). To test this hypothesis many recent papers have used in vitro models in which the transport of microtubules along axons can be visualized during brief periods of growth. Several have used approaches in which tubulin conjugated to a fluorophore was introduced into neurons, allowed to distribute along the axons, and then discrete zones of axonal microtubules were labeled by photobleaching or photoactivation and tracked during axon growth. Results in a variety of model systems indicated that the photolabeled zones did not move along the axon during growth, suggesting that the en mass movement of microtubules could not be the vehicle of tubulin axoplasmic transport (Keith, 1987; Lim et al., 1989, 1990; Okabe and Hirokawa, 1990, 1992; Keith and Farmer, 1993; Sabray et al., 1995; Takeda et al., 1995). While photolabeled microtubules have been observed to move anterograde in growing Xenopus axons, the observed movements tend to be slower than axon elongation (Reinsch et al., 1991) and are likely due to the pulling of the axon by the growth cone rather than the axoplasmic transport (Okabe and Hirokawa, 1992; Sabry et al., 1995; Takeda et al., 1995).

Experiments with cultured rat sympathetic neurons indicated that axon elongation occurred in the presence of low concentrations of vinblastine given to prevent microtubule polymerization. This suggests that the growing axons must have been supplied by the transport of microtubules assembled before vinblastine treatment (Baas and Ahmad, 1993; Yu et al., 1996). However, Miller and Joshi (1996) have challenged this interpretation, showing that low concentrations of vinblastine inhibit axon growth without eliminating the polymerization of fluorescent-labeled tubulin injected into the cell bodies. They observed that vinblastine did reduce polymerization of microtubules to

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levels low enough to allow individual labeled microtubules to be visualized by fluorescence microscopy. At 30 min after injection they observed labeled microtubules concentrated in the cell bodies and near the growth cones, but absent from the intervening axon shaft. Other aspects of the distribution of labeled microtubules and the absence of transport of fluorescent, stabilized microtubules injected into the cells, suggest that microtubules cannot be the transport form of tubulin in rat sympathetic neurons. While fluorescent-labeled microtubules injected into squid giant axon are anterogradely transported (Terasaki et al., 1995), it has been argued that this result may reflect the transport of short microtubule fragments by a nonspecific transport mechanism that transports a variety of objects measuring 500 nm or less (Miller and Joshi, 1996).

A novel approach to this question was developed by Funakoshi et al. (1996). They microinjected caged fluorescein-labeled tubulin into dorsal root ganglion neurons newly cultured from adult mice. After 10–20 h of incubation, they photobleached segments of the axons, allowed 1–20 min after photobleaching for tubulin transport to occur, and processed the cells for immunoelectron microscopy using an anti-fluorescein primary antibody and a gold-conjugated secondary antibody to visualize the bleached tubulin. No labeled tubulin was detected outside of the photobleached regions in cells that were permeabilized to release unpolymerized tubulin indicating that photobleached microtubules were not transported along the axons during the observation period. However, when cells were not permeabilized so that tubulin subunits would be retained, labeled tubulin was present both proximal and distal to the photobleached regions with distal axon segments displaying between 1.48–2.28-fold more label than proximal segments. The assymetric distribution rules out diffusion as a mechanism and indicates that tubulin subunits were transported from the bleached region preferentially in the anterograde direction.

The model systems used to study tubulin transport along axons during growth have been subject to several limitations. In most experiments, only the movement of the microtubule fraction of tubulin was visualized, not the subunit fraction. Because of exchange of unlabeled for labeled tubulin subunits during normal microtubule turnover, observations have been limited to short periods during which only small amounts of growth can occur. Studies have been limited to the early phase growth when axons are short and not highly branched.

We felt that a different approach, not subject to these limitations, may contribute to discovering the mechanisms by which tubulin is supplied to a highly branched axonal arbor growing a long distance from the cell body. We used compartmented cultures in which sympathetic neurons from newborn rats plated in a center compartment extended their axons left and right, under silicone grease barriers, and into separate distal compartments. We labeled tubulin during a pulse of [^{35}S]methionine, and separated tubulin from other proteins by immunoprecipitation with monoclonal antibodies to α and β tubulin. Because of this, we were able to follow the transport of labeled tubulin from the cell bodies and proximal axons into the distal axons. Since neurons were the only cells present in the cultures, all of the tubulin detected was synthesized by them. Furthermore, our method provided a measure for all of the tubulin made by the neurons during the pulse regardless of whether it was in microtubule or subunit form. Also, since sympathetic neurons display axonal elongation rates of ~1 mm/d while producing an extensively branched axonal arbor (Campenot, 1982*a*), we were able for the first time, to follow the axoplasmic transport of tubulin over many days during which substantial axon growth occurred.

Materials and Methods

Compartmented Cultures

Superior cervical ganglion neurons were obtained from newborn rats (Sprague-Dawleys supplied by the University of Alberta Farm, Edmonton, AB, Canada), and plated in the center of compartmented dishes as previously described (Campenot et al., 1991). The compartmented dishes (Campenot, 1992) consisted of a Teflon divider (Tyler Research Instruments, Edmonton, AB, Canada) seated with silicone grease (Dow Corning, Midland, MI) into a collagen-coated 35-mm Falcon tissue culture dish. Dividers which partitioned the dishes into 3 or 5 compartments were used. In 3-compartment cultures the cell bodies/proximal axons reside in a 1.5-mm-wide center compartment, axons cross 1.0-mm-wide partitions at the left and right and elongate within left and right distal compartments (See Fig. 1, *A* and *B*). The neuronal cell bodies form clusters in the middle of the center compartments such that the nearest cell bodies are at least 0.2 mm from the barrier, and most cell bodies are farther away. Therefore, tubulin must travel through at least 1 mm of axon before emerging into the distal axons in the left and right compartments. The travel distance is generally >1 mm because most cell bodies are farther away and axons do not traverse a perfectly straight course.

In 5-compartment cultures, the cell bodies/proximal axons reside in a 1.5-mm-wide center compartment, axons cross 1.0-mm-wide partitions at the left and right, and elongate within 4.4-mm-wide intermediate compartments; then, they cross another 1.0-mm barrier at the left and right to elongate in left and right distal compartments (see Fig. 1, *C* and *D*). Therefore, the minimum travel distance between the closest cell bodies and the distal compartments is 6.6 mm.

Culture Media

Ll5 medium without antibiotics (Gibco BRL, Gaithersburg, MD) was supplemented with the additives prescribed by Hawrot and Patterson (1979) including bicarbonate and methylcellulose. In 3- and 5-compartment cultures, rat serum (2.5%, provided by the University of Alberta Laboratory Animal Services, Edmonton, AB, Canada) and ascorbic acid (1 mg/ml) were supplied only in medium given to the center compartments containing the cell bodies. Culture medium was changed each week. Nonneuronal cells were eliminated by supplying 10 μM cytosine arabinoside in the center compartments during the first 7 d. 2.5S nerve growth factor (NGF)[1] (Cedarlane Laboratories Ltd., Hornby, ON, Canada) was initially supplied in center compartments at 10 ng/ml to allow cell survival and neurite outgrowth.

Medium in distal compartments of 3-compartment cultures was supplied with 100 ng/ml NGF (200 ng/ml in a few experiments) throughout the experiments to promote establishment of a dense growth of axons. After 7 d in culture, NGF was discontinued in the center compartment to confine subsequent axon growth to the NGF-containing side compartments (Campenot 1982*a*, 1987).

In 5-compartment cultures intermediate compartments were supplied with 10 ng/ml NGF to allow axons to cross, and distal compartments were supplied with 100 ng/ml NGF to promote high density growth. Between 8–14 d in culture NGF was withdrawn from the center and intermediate compartments to confine subsequent axon growth to the NGF-containing distal compartments.

Metabolic Labeling

Cultures were metabolically labeled by incubation of the cell bodies in the center compartment with a labeling medium which consisted of culture

1. *Abbreviations used in this paper*: IPB, immunoprecipitation buffer; NGF, nerve growth factor.

medium supplied with *trans*-label containing 250 μCi/ml [^{35}S]methionine (ICN Pharmaceuticals, Montreal, QC, Canada) and with unlabeled methionine reduced to 10% of our standard culture medium. During labeling, the distal (and intermediate compartments in 5-compartment cultures) were supplied with medium containing 10% of normal methionine.

Ordinarily, the medium in the center compartments is confluent with ~1.5 ml of medium in the perimeter of the dish. For the radioactive incubations, this medium was removed, and most residue was aspirated from the center slot with a gel-loading-style micropipette tip. Then, 40 μl of labeling medium was pipetted into the slot where it would remain without running out into the dish perimeters. At the end of the pulse, the labeling medium was aspirated from the slot, and the slot and the perimeter of the dish were filled with 1.5 ml of standard medium. This effectively diluted any residual label, effectively terminating the pulse (Fig. 5).

Harvesting and Tubulin Immunoprecipitation

All procedures previous to the addition of SDS sample buffer were carried out on ice. Culture medium was aspirated from all compartments (using a gel-loading-style micropipette tip for the center compartment) and replaced with Tris-buffered saline (TBS; 20 mM Tris-HCL, 137 mM NaCl, pH 7.5) to rinse. Complete immunoprecipitation buffer (IPB) was made fresh by adding 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mM sodium orthovanadate to IP stock buffer (TBS, 10% glycerol, 1% NP-40). TBS was aspirated from all compartments, replaced with 40 μl IPB, and the dishes were incubated 20 or more minutes on a slow rocker. Distal axons were harvested first, intermediate axons second (in 5-compartment cultures), and cell bodies/proximal axons were harvested last. Harvesting was accomplished by scraping any residual cellular material from the substratum with a gel-loading-style micropipette tip. Then the same tip was attached to a Pipetteman and used to collect the sample. Samples within a single experimental treatment were pooled into a single microfuge tube. Samples contained material from 2–6 cultures depending upon the experiment. They were stored at −70°C.

For immunoprecipitation, 5 μl mouse anti–α-tubulin (Sigma; No. T-9026) monoclonal antibody and 10 μl mouse anti–β-tubulin monoclonal antibody (Sigma; No. T-4026) were added to a clean centrifuge tube for each sample. This was accomplished by mixing together the appropriate volumes of antibodies and then distributing them to clean tubes to ensure that each sample received the same amount of antibodies.

The volumes of the thawed samples were equalized by addition of fresh IPB to a maximum volume of 1 ml. The samples were triturated with a 3-ml syringe and a 22-g needle to disperse remaining cell debris, microfuged for 2 min to spin down cell debris, and supernates transferred to the antibody-containing microfuge tubes. The tubes were incubated for 1 h on ice and then 25 μl of protein A/G–Agarose beads (Santa Cruz Biotechnology; No. SC-2003) was added to each sample. The samples were incubated on a rotator in a cold room for 2 h. Samples were microfuged for 1 min, the supernatant discarded, and the pellet washed 3× by resuspending in 1 ml of IPB followed by a 1-min spin. After the last spin 100 μl of SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, 0.001% bromophenol blue) was added to each sample, the samples were boiled, and then run on SDS-PAGE containing 8% acrylamide.

Harvesting Efficiency

To evaluate the efficiency of harvesting of radiolabeled tubulin from the culture dishes, extracts were collected from the cell bodies/proximal axons and distal axons in the usual manner from cultures in two experiments, one involving a 24-h pulse followed by immediate harvest and one involving a 24-h pulse, 4-d chase, and harvest. Then the cultures were harvested a second time with fresh IP buffer to determine if any residual tubulin remained. Some tubulin was harvested in the second samples, but the first samples contained between 81 and 92%. The total tubulin harvested with the first samples averaged 87% in the cell bodies/proximal axons and 86% in the distal axons.

In other experiments we compared a cold, calcium extraction procedure for solubilizing tubulin (0.1 M Pipes, pH 6.9, 1 mM $MgSO_4$, 5 mM $CaCl_2$, 20 min on ice; Black et al., 1986) with our IP buffer extraction. In experiments involving chase periods up to 5 d, both procedures recovered closely similar amounts of tubulin suggesting that the IP buffer extraction was not leaving behind significant radiolabeled tubulin in the cell debris pellet. To directly test this, cell debris pellets remaining after IP buffer extraction of cultures radiolabeled for 20 h were dissolved directly in SDS sample buffer. The pellet extracts were run side by side on SDS-PAGE with the solubilized tubulin immunoprecipitates. SDS-dissolved pellets displayed radioactivity at the apparent molecular weight of tubulin which was only 9–12% of the radioactivity in the IP buffer fraction. In another experiment, the cell debris pellets remaining after IP buffer extraction were extracted with cold calcium buffer, and the remaining cell debris pellets were dissolved in SDS sample buffer. In this experiment 94–95% of total radiolabeled tubulin was recovered in the IP buffer extract. The cold calcium buffer recovered the remaining 5–6%, and the pellet was not above background. These results indicate that the IP buffer extraction procedure used in these experiments recovered ~90% or more of the radiolabeled tubulin in the cellular material.

Calibration Curve

To check for accurate quantitation of our results, we pulse-labeled six cultures with [^{35}S]methionine for 24 h. The cell body/proximal axons were kept separate from the distal axons, but the extracts from all the cultures were pooled and divided into three aliquots of different volumes corresponding to the extract from one, two, or three cultures. Volumes were then equalized with IP buffer and immunoprecipitated with the standard antibody concentration. Radioactivity quantified by phosphorimaging ranged from a low of 1020 up to 5483 which was about equal to the highest values obtained in the experiments in this paper. For both the cell bodies/proximal axons and the distal axons the relationships between extract volume and tubulin were highly linear with R^2 greater than 0.90. The threefold increase in extract resulted in a 4.6-fold increase in the tubulin measured in the cell body/proximal axons, and a 3.6-fold increase in the tubulin measured in the distal axons. Therefore, the methods used were sufficiently quantitative to evaluate differences in tubulin measured in these experiments.

SDS-PAGE, Fluorography, and Phosphorimaging

Samples were run on SDS-PAGE containing 8% acrylamide. The gels were fixed in 50% methanol/20% acetic acid for 10–15 min, rinsed briefly with double distilled water and incubated in 1 M Na^+ salicylate (pH 7.5) containing 1 μl/100 ml β-mercaptoethanol, and then dried. Gels were exposed on phosphorimager plates overnight and the radioactivity in the tubulin bands was quantified by phosphorimaging with a BAS 1000 Bio-Imaging Analyzer and MacBAS software (Fujix, Tokyo). Fluorograms were made by exposing the gels on Kodak X-OMAT AR film at −70°C for 3–5 d.

Results

Compartmented Cultures

In most experiments, cultures were used in which neurons elaborated large axonal trees in distal compartments. To accomplish this, sympathetic neurons from newborn rats were plated into the center compartments of 3-compartment dishes (Fig. 1, *A* and *B*) with 2.5S NGF provided in the center, proximal compartments at 10 ng/ml and in the left and right compartments at 100 ng/ml (sometimes 200 ng/ml). Axons were well established in the distal compartments within 2 or 3 d after plating. By 7 d, growth in the distal compartments was luxuriant and extensive. At this time NGF was withdrawn from the proximal compartments to terminate growth there, but was supplied for the duration of the experiments at 100 ng/ml in the distal compartments where the distal axons continue to grow for at least several weeks.

Time Course of Tubulin Transport into Distal Axons

To determine the time course of the transport of newly synthesized tubulin into distal axons, 11-d-old, 3-compartment cultures of sympathetic neurons were pulsed for 24 h with ^{35}S-labeling medium. Three cultures were harvested

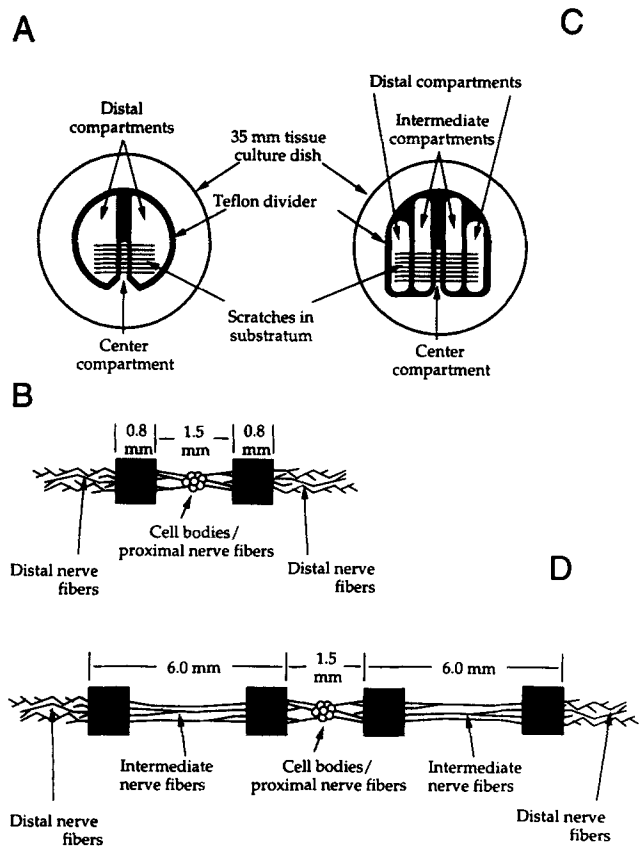

Figure 1. Compartmented cultures. *A* shows a schematic diagram of a 3-compartment culture consisting of a 35-mm tissue culture dish divided into left and right distal compartments and a narrow center compartment which connects with the medium in the dish perimeter. The axons of sympathetic neurons plated in the center compartment grow along 20 tracks made by scoring the collagen-coated dish floor with a pin rake before seating the Teflon divider with silicone grease. The axons grow under the silicone grease and enter the distal compartments at the left and right. *B* shows a schematic diagram of neurons on a single track in a 3-compartment culture. Black squares represent the Teflon divider under which the axons cross. *C* shows a schematic diagram of a 5-compartment culture. The details are similar to *A* except that the axons traverse an intermediate compartment before reaching the distal compartment. *D* shows a schematic of neurons on a single track in a 5-compartment culture.

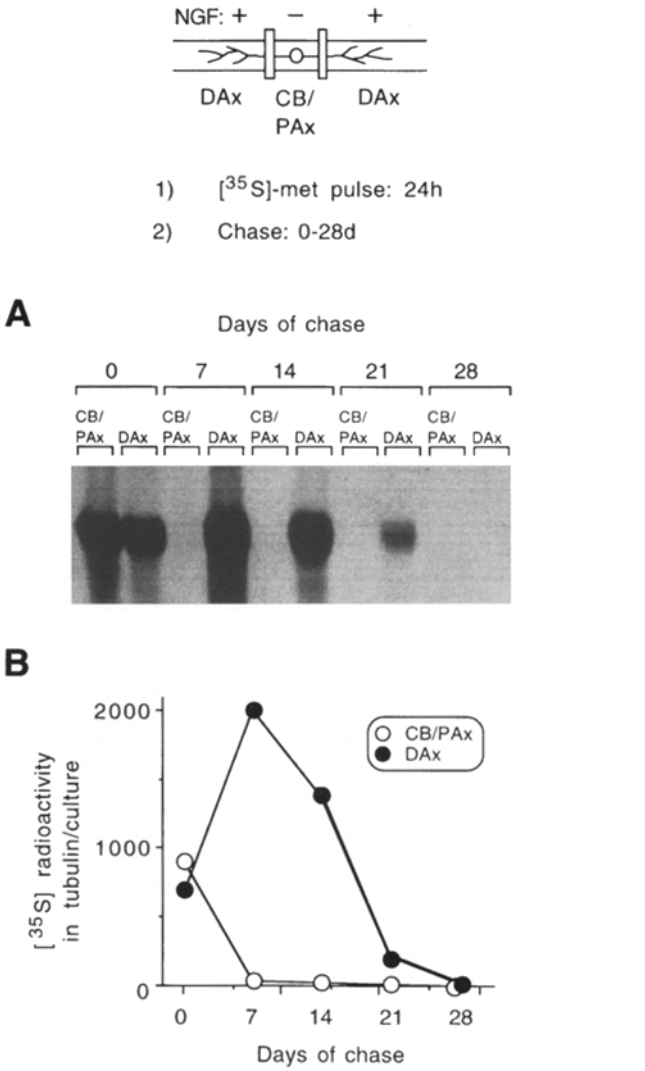

Figure 2. Time course of tubulin transport into distal axons. 11-d-old sympathetic neurons in compartmented cultures were pulsed for 24 h with [^{35}S]methionine in the center compartments. Three cultures were harvested for tubulin immunoprecipitation immediately after the pulse and three more each week for 4 wk. The distal axons in left and right compartments were harvested and pooled, and then the cell bodies and proximal axons were harvested. *A* shows a fluorogram of an SDS gel of samples obtained by immunoprecipitation of the cell extracts with a mixture of anti-α- and β-tubulin monoclonal antibodies. Whether the sample originated from cell bodies and proximal axons (CB/PAx) or distal axons (DAx), is indicated above each lane. *B* shows a plot of phosphorimager scans made of the tubulin bands of the gel in *A*. Open circles represent cell bodies and proximal axons (CB/PAx) and filled circles represent distal axons (DAx).

at the end of the 24-h pulse (chase time = 0) and then at 1-wk intervals for 4 wk. Distal axons in left and right compartments were harvested first, followed by the cell bodies and proximal axons in the center compartments. Cell extracts were stored at −70°C. The cultures not harvested were given fresh medium each week. After extracts were collected from the last cultures, α and β tubulin were immunoprecipitated from all the extracts. The radioactivity of the tubulin bands on SDS-PAGE was quantified with a phosphorimager.

At the end of the 24-h pulse, half of the radiolabeled tubulin had already moved into the distal axons, and after 7 d all of the detectable tubulin had moved into the distal axons (Fig. 2). Note that the total radioactivity in tubulin after a 7-d chase was about the same as at 0 chase, indicating that little, if any, tubulin was degraded during the first week after synthesis. The half-life of tubulin in the neurons was between 2 and 3 wk.

We replicated this time course experiment, but with a variation in procedure. The cultures used were 32-d-old at the start of the experiment, and immediately before starting the labeling pulse, we axotomized the cultures by removing the axons from the left and right compartments with a jet of sterile, distilled water delivered by syringe through a 22-g needle. Distal axons reliably regenerate af-

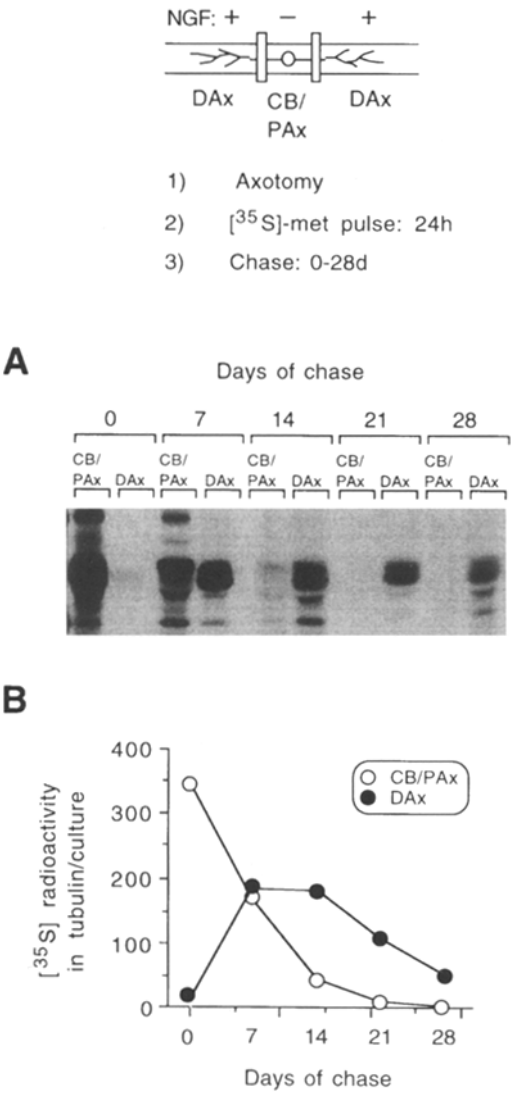

Figure 3. Time course of tubulin transport into regenerating distal axons. Distal axons of 32-d-old cultures were axotomized immediately before the start of a 24-h labeling pulse. Otherwise the experiment was the same as in Fig. 2, *A* and *B* are as in Fig. 2.

ter this procedure (Campenot, 1982*a*). As in the previous experiment, three cultures were harvested immediately after the pulse for the 0-chase time point, and three cultures were harvested each week for the ensuing 4 wk.

Labeled tubulin was detected in the cell bodies/proximal axons at the end of the 24-h pulse, but little or no labeled tubulin was present in the distal compartment extracts (Fig. 3). This was expected since axonal regeneration produces relatively little axonal material in the distal compartments during the first 24 h. After 1 wk about half of the labeled tubulin had moved into the regenerated distal axons. As in the intact neurons, the total radioactivity in tubulin did not change during the first week. By 2 wk the distal axons contained most of the remaining labeled tubulin. As in the case of intact neurons, the half-life of tubulin was between 2 and 3 wk.

The experiment with intact cultures (Fig. 2) yielded the surprising result that nearly half of the tubulin synthesized during a 24-h pulse had already moved into distal axons by the end of the pulse, and the vast majority had moved into distal axons within a week. During the course of our tubulin studies, many experiments included groups of cultures in which 24-h labeling pulses were followed either by immediate harvesting or chase periods of 1–7 d (Fig. 4). The phosphorimager data from the 11 groups given no chase displayed an average of 41% (±13 SD) of newly synthesized tubulin already transported into distal axons by the end of the 24-h pulse. The cultures ranged in age between 11 and 37 d, but there was no relationship between the percent of tubulin transported and culture age ($R^2 = 0.004$). After 2 d of chase ~90% of the labeled tubulin had moved into distal axons (Fig. 4).

Tubulin Transport after a 6-h Pulse

Experiments involving shorter pulses were used to further investigate how rapidly tubulin moves into distal axons. The results of two experiments are shown in Fig. 5. Radiolabeled tubulin was detected in distal axons immediately at the termination of a 6-h labeling pulse (6% in Fig. 5 *A*

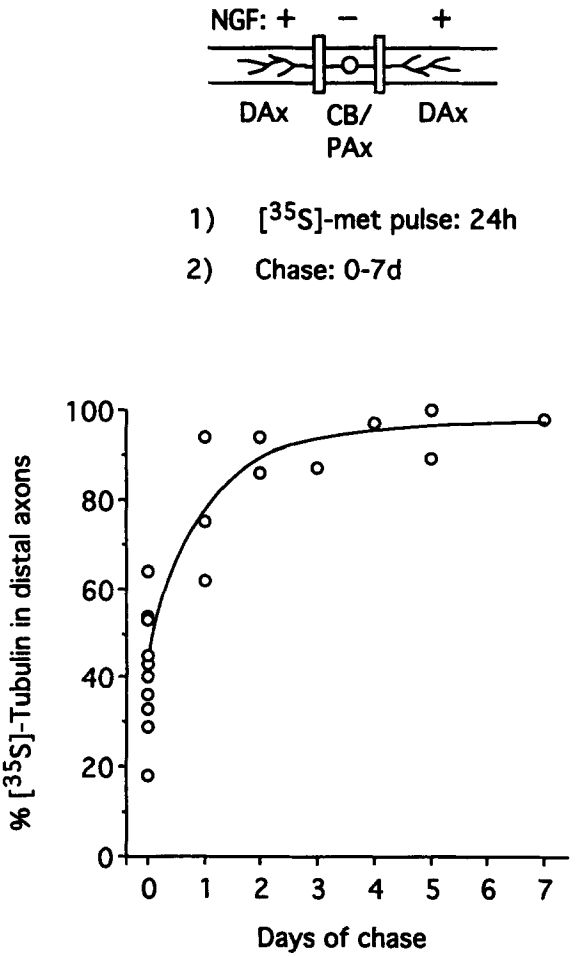

Figure 4. Tubulin distribution after 0–7 d of chase. Compilation of data from 21 groups of cultures in which a 24-h ^{35}S-labeling pulse was followed by either no chase or 1–7 d of chase. Phosphorimager scans were used to calculate the percent of the total labeled tubulin that was transported into distal axons. The curve was fit by eye.

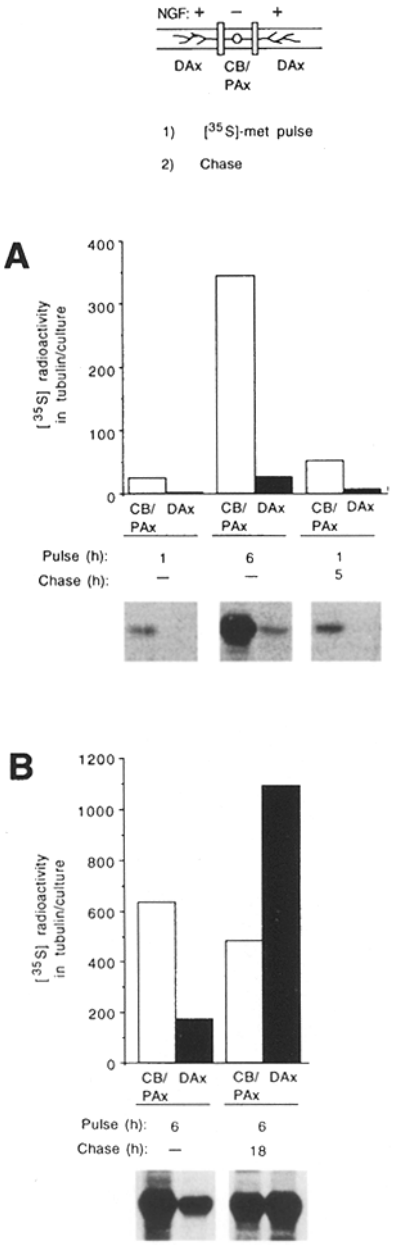

Figure 5. Tubulin transport into distal axons at early times. Phosphorimager scan data of immunoprecipitated α and β tubulin from two experiments are shown along with fluorograms of the tubulin bands. In *A*, 3 groups (2–3 cultures/group) of 12-d-old cultures were pulse labeled for 1 h and harvested (*left pair of bars*); pulse labeled for 6 h and harvested (*center pair of bars*); or pulse labeled for 1 h, followed by a 5-h chase (*right pair of bars*), and then harvested. Open bars represent the radioactive tubulin in the cell bodies and proximal axons (CB/PAx) and filled bars represent the radioactive tubulin in the distal axons (DAx). The corresponding tubulin bands are shown beneath each bar. In *B*, two groups (six cultures each) of 21-d-old cultures were pulse labeled for 6 h and harvested; or pulse labeled for 6 h, chased for 18 h, and then harvested. Details are as in *A*.

and 22% in Fig. 5 *B*). Labeled tubulin was detected in cell bodies/proximal axons after a 1 h-pulse, but none had moved into the distal axons (Fig. 5 *A*). To check the effectiveness of our chase protocol (see Materials and Methods), a group of cultures was pulsed for 1 h followed by a 5-h chase (Fig. 5 *A*) The total labeled tubulin in this group was much less than after a 6-h pulse, showing that the chase effectively terminated most of the additional labeling of tubulin. However, the total labeling in the 1-h pulse 5-h chase group was somewhat higher than in the 1-h pulse group suggesting that some further incorporation of label into tubulin occurred during the chase. This likely reflects the incorporation of residual intracellular [^{35}S]methionine. When a 6-h pulse was followed by an 18-h chase, 70% of the labeled tubulin had moved into distal axons (Fig. 5 *B*). The increase in total radioactivity in the 18-h chase group may reflect incorporation of residual intracellular [^{35}S]methionine during the chase period.

Since the percent of radioactivity transported by the end of a 6-h pulse was small, we wanted to make certain that it was transported there by the axons. Experiments were conducted in which distal axons in the left compartments were axotomized immediately before the start of a 6-h pulse while axons in right compartments remained intact. Extracts were harvested at the end of the pulse. Fig. 6 shows that relatively little radioactive tubulin (3% of total) was detected in left compartments compared with the right compartments (12%). This experiment was repeated with virtually identical results of 4% of total tubulin on the axotomized side vs 17% on the intact side. These results indicate that the radiolabeled tubulin in the right compartments after 6 h was transported there by the axons.

In the compartmented cultures used in these experiments, tubulin must travel through ~1 mm of proximal axons before reaching the distal compartments (Fig. 1 *B*). The results indicate that tubulin can be synthesized in the cell body and travel at least 1 mm along the axon within 6 h. This establishes a minimum velocity for tubulin of 4 mm/d along the axons. Since some of the tubulin detected in distal compartments after 6 h has likely traveled more than 1 mm, the velocity of tubulin in these axons is likely to be faster.

We also wanted to determine how much tubulin would travel through an axon longer than 1 mm and continue moving towards the terminals. We used 5-compartment cultures in which intermediate compartments interposed a distance of 6 mm between the cell bodies and the distal axons. At the end of a 6-h pulse, 97% of labeled tubulin remained in the center compartment (Fig. 7). At the end of a 24-h pulse, labeled tubulin was detected in intermediate (12%) and distal (4%) axons indicating a minimum transport rate of at least 6 mm/d, somewhat faster than the minimum velocity indicated in the experiments with 3-compartment cultures. After a 24-h pulse followed by a 5-d chase, 10% of the remaining labeled tubulin was localized in the cell bodies/proximal axons, 15% resided in the intermediate axons, and 76% had passed through the intermediate axons and resided in the distal axons. The 24-h pulse followed by a 5-d chase was repeated in another experiment yielding 19% of the labeled tubulin remaining in the cell bodies/proximal axons, 8% in the intermediate axons, and 76% in the distal axons.

Discussion

These results reveal several important features of tubulin

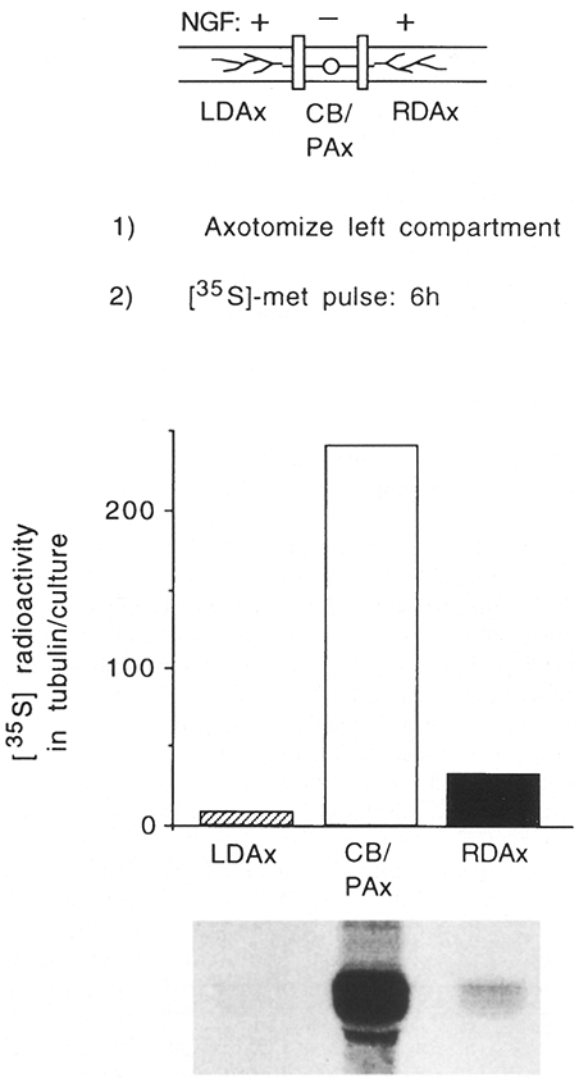

Figure 6. Tubulin production/distribution after a 6 h-pulse preceded by left axotomy. A single group of five cultures was used at 28 d of age. Immediately before a 6-h labeling pulse, axons were removed from left compartments to eliminate axonal transport of tubulin. Phosphorimager scan data of immunoprecipitated tubulin from left (LDAx), center (CB/PAx), and right (RDAx) compartments are shown along with fluorograms of the tubulin bands below the corresponding bars.

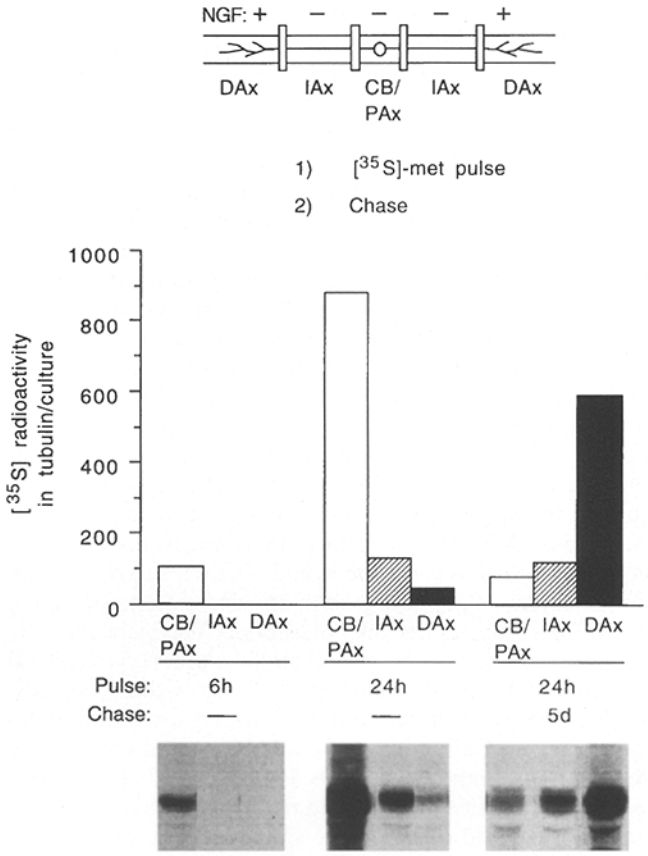

Figure 7. Tubulin production/distribution in 5-compartment cultures. Phosphorimager scan data of immunoprecipitated tubulin from an experiment with 5-compartment cultures is shown along with fluorograms of the corresponding tubulin bands. In these cultures there were intermediate compartments through which intermediate axons (IAx) passed producing a 6-mm distance between cell bodies and proximal axons in the center compartments and axons in the distal compartments. Cultures were 20-d-old, and four were used per group. Three groups of cultures were either pulse labeled for 6 h and harvested (*left three bars*); pulse labeled for 24 h and harvested (*center three bars*); or pulse labeled for 24 h, chased for 5 d, and harvested (*right three bars*).

production and transport. First, virtually all of the radiolabeled tubulin present at the termination of a 24-h pulse remained present 1 wk later (Figs. 2 and 3) suggesting that there is little degradation of tubulin during the first week after synthesis. Thus, the radiolabeled tubulin present at the end of a 24-h pulse represents tubulin production rather than a balance between production and degradation.

Second, ~40% of tubulin produced during a 24-h pulse in 3-compartment cultures had been transported into distal axons by the end of the pulse, ~75% of the tubulin had been transported within 1 d of chase, and ~90% had been transported within 2 d of chase. This indicates that newly synthesized tubulin is efficiently delivered to the distal axons, and that the bulk of the newly synthesized tubulin is transported at velocities exceeding 1 mm/d. Tubulin was also delivered efficiently to more distant axons in 5-compartment cultures; 75% of the tubulin pulse passed through at least 6 mm of axons to reach the distal compartments within 5 d.

Third, the observation made in 3-compartment cultures that radiolabeled tubulin crosses a 1-mm barrier within 6 h of the onset of the labeling pulse indicates that the leading edge of the tubulin pulse was transported along the axons at a velocity greater than 4 mm/d. Furthermore, in 5-compartment cultures radiolabeled tubulin traversed 6 mm to reach distal compartments within 24 h of pulse onset indicating a leading edge velocity of at least 6 mm/d and further indicating that the leading edge of the tubulin pulse did not slow with distance from the cell body. These results are consistent with the velocity of 4 mm/d reported for tubulin transport in mammalian peripheral nerve (Hoffman and Lasek, 1980; McQuarrie et al., 1986). The present experiments were not designed to directly observe whether

labeled tubulin reaches growth cones. However, the observation that the transport velocity is maintained across 6 mm of axon suggests that the leading edge of labeled tubulin maintains a high velocity all along the axons. Axons of sympathetic neurons in compartmented cultures elongate at 1 mm/d while producing a highly branched axonal tree (Campenot, 1982*a*). Traveling at a velocity exceeding 4 mm/d, tubulin starting out in a 5-mm-long axon will reach the growth cones within 2 d. Starting out in a 30-mm-long axon, the growth cones will be reached within 10 d. Since little tubulin is broken down within the first week after synthesis and the half-life exceeds 2 wk, the growth cones of sympathetic neurons in these experiments would be easily reached by newly synthesized tubulin. The axon lengths in these experiments equal or exceed the lengths of sympathetic axons in vivo during embryonic and postnatal development. Therefore, our results suggest that the tubulin transport in developing rat sympathetic neurons is capable of delivering tubulin synthesized in the neuronal cell body with sufficient speed and efficiency to provide an ample supply to support growth anywhere along the axon. Therefore, our results do not support the view that the transport of tubulin (and presumably other cytoskeletal constituents) from the cell body to the growth cone necessarily imposes a rate limitation on axon growth during development. Tubulin transport could be rate limiting during regeneration in the adult where longer distances can intervene between the cell body and regenerating axons and regeneration could occur before sufficient time has elapsed for newly synthesized tubulin to reach the growth cones (Hoffman and Lasek, 1980).

Diffusion cannot be the mechanism of the tubulin transport described in these experiments. In the case of 3-compartment cultures the distance to reach distal compartments was ~1 mm, and in 5-compartment cultures the distance was more than 6 mm. Both greatly exceed the 100-μm limit for diffusion calculated by Reinsch et al. (1991).

It is highly unlikely that the transport of microtubules can account for the magnitude and velocity of tubulin transport that we observed. This can be best appreciated by comparing our results with those obtained in photoactivation experiments with Xenopus, the model most supportive of the microtubule transport hypothesis. The rate of axon elongation observed in Xenopus axons (Reinsch et al., 1991) was comparable to the 1 mm/d axon elongation rate observed in sympathetic axons (Campenot, 1982*a*). In Xenopus axons the transport of microtubule photoactivation zones near the growth cones tended to approach the rate of growth cone advance, while photoactivation zones in more proximal regions of the axons, back from the growth cones, tended to move slowly. The only examples of photoactivation zones in Xenopus axons that moved more rapidly than growth cones occurred in cases where growth cone advance was particularly slow (Reinsch et al., 1991). The movements of photoactivation zones in Xenopus axons have been reinterpreted as likely due to the pulling of the axon by the growth cone rather than axoplasmic transport of microtubules (Okabe and Hirokawa, 1992; Sabry et al., 1995; Takeda et al., 1995). In any event, in the present experiments the front of newly synthesized tubulin was transported at a velocity exceeding 4 mm/d, more than four times the rate of axon elongation, and much faster than the rate of microtubule transport observed in Xenopus or any other system.

Some general considerations also suggest that translocation of microtubules cannot account for the transport of newly synthesized tubulin in rat sympathetic neurons. It is a well established principle that the cross section of a proximal axon is much smaller and contains many fewer microtubules than the combined cross section of its distal branches; Zenker and Hohberg (1973) found that the combined cross section of all terminal fibers of A-α-motoneurons contained 11 times as many microtubule profiles as the stem axon. Although microtubules can be transported into growing sympathetic axons (Baas and Ahmad, 1993; Yu et al., 1996), even if the entire microtubular cytoskeleton of the proximal sympathetic axons translocated at 4 mm/d, it could only supply sufficient tubulin for a few branches of distal axon elongating at 1 mm/d. It is highly unlikely that the extensive axonal arbors of sympathetic neurons could be supplied with sufficient tubulin by a microtubular transport mechanism, even if the microtubules were transported at an unprecedented velocity exceeding 4 mm/d. Thus, our results suggest that the transport form in which tubulin is supplied to rapidly growing axons of rat sympathetic neurons is likely to be some kind of subunit such as a heterodimer or an oligomer considerably smaller than a microtubule.This conclusion is consistent with the conclusions of Miller and Joshi (1996) and the direct observations of nonmicrotubule tubulin transport by Funakoshi et al. (1996).

Some previous evidence is relevant to the issue of the transport form of tubulin. Results from experiments with cultured rat sympathetic neurons suggest that tubulin in neurons exists in subunit form, or polymerized into relatively labile microtubules, or polymerized into relatively stable microtubules (Baas and Black, 1990; Brown et al., 1992). In rat sympathetic neurons 80% of metabolically labeled tubulin polymerized into microtubules within a few hours of synthesis (Black et al., 1986), and 50% of biotinylated tubulin injected into the cell bodies polymerized into microtubules within 2.2 h (Li and Black, 1996). Most of the tubulin in these experiments were polymerized before transport into distal axons was complete, and results like these have been interpreted as indicating that the tubulin must be transported in microtubule form. However, tubulin can have a short residence time in microtubules, moving back and forth between microtubules and the cytosol several times as the tubulin moves along the axon. Therefore, whether the tubulin is transported while in microtubule form, while in subunit form, or both cannot be readily determined from composition data.

Moreover, in these previous experiments with cultured sympathetic neurons, the entire surfaces of the neurons were bathed in medium containing NGF, and the results may not be directly applicable to compartmented cultures since we withdrew NGF from the cell bodies/proximal axons (and intermediate axons in 5-compartment cultures) in order to terminate axon growth in these compartments and concentrate growth at the distal axons (Campenot, 1982*a*,*b*). Our results indicated that the vast majority of labeled tubulin passed through these axons and entered into the growing distal axons. It is a reasonable speculation that polymerization of tubulin in sympathetic neurons may be

regulated by NGF and may be more active in distal regions of the axons that are growing in response to NGF than in proximal regions of the axons without NGF in the medium bathing them. It is, therefore, possible that polymerization of much of the newly synthesized tubulin subunits into stable microtubules may have been delayed in compartmented cultures until the tubulin reached the distal axons and growth cones exposed to NGF. This interpretation is supported by the finding of Miller and Joshi (1996) that at 30 min after injection of fluorescent tubulin into newly plated rat sympathetic neurons, labeled microtubules were observed concentrated in the cell bodies and especially near the growth cones, but were absent from the intervening axon shaft.

Finally, the present results suggest that the compartmented culture system, with its many advantages for control and analysis, offers a promising in vitro model in which to explore the mechanisms and regulation of axonal transport of tubulin and other slow-transported molecules.

We thank Grace Martin for technical assistance with cell culture, and Russell Watts for technical assistance with the experiments. We are grateful to Ellen Shibuya, Daren Ure, Dennis Vance, and Jean Vance for helpful discussions and comments on the manuscript. R.B. Campenot is an Alberta Heritage Foundation Senior Scholar and an Associate Investigator of the NeuroScience Network of the Canadian Networks of Centers of Excellence.

D.L. Senger is supported by a studentship from the NeuroScience Networks. Financial support for this work was supplied by the Medical Research Council of Canada.

Received for publication 5 July 1996 and in revised form 22 August 1996.

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