Membrane Biogenesis in the Sprouting Neuron. I. Selective Transfer of Newly Synthesized Phospholipid into the Growing Neurite

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ABSTRACT Our goal was to elucidate the pathway of newly synthesized phospholipid into the growing neurite. This was accomplished in pulse-chase studies with the phospholipid precursor [^{3}H]glycerol, using sprouting explant cultures of rat superior cervical ganglion as an experimental system. After the pulse with the precursor and various chase periods, we separated perikarya and neurites microsurgically and extracted their phospholipids. The phospholipid extract from the perikarya exhibited a steep rise followed by a rapid decline in specific radioactivity. In contrast, an increase in neuritic specific radioactivity of phospholipid was observed only after a lag period of ~60 min. Nearly quantitative transfer of newly synthesized phospholipid from the perikarya into the neurites could be demonstrated. Both the decline in perikaryal specific radioactivity and the increase in its neuritic counterpart, i.e., the proximodistal transfer, could be blocked with the microtubule drug colchicine and the metabolic uncoupler, 2,4-dinitrophenol. These observations indicate preferential export of newly synthesized phospholipid from the perikaryon (the major or exclusive site of synthesis) into the growing neurites, most likely by rapid axoplasmic transport of formed elements.

The developing, sprouting neuron is characterized by vectorial growth of its processes and very rapid net expansion of its plasmalemma, at a rate of ~0.5 μm^2/min per neurite (neurons of the mammalian peripheral nervous system; see reference 17). This system offers the interesting possibility of studying the intracellular pathways(s) of newly synthesized membrane components from their site of synthesis to their point of insertion into the plasma membrane. In such a highly polarized system, incorporation into the plasmalemma of proteins and lipids could occur predominantly in a distal region (at the growth cone), it could occur preferentially at the perikaryon, or else it could occur at random. To gain insight into this problem, we used tritiated glycerol as a precursor for membrane phospholipid in pulse-chase experiments with cultured neurons. After chase, neuritic sprouts were separated from neuronal perikarya, and specific radioactivity of phospholipid was analyzed in the two portions of the neuron. Thus, transfer of newly synthesized phospholipid from the perikaryon to the neurite could be measured. A preliminary report on this work has been presented in abstract form (15).

MATERIALS AND METHODS

Tissue Culture: The experiments were carried out on explant cultures of rat superior cervical ganglia, removed just before, or right after, birth of the pups. The ganglia were stripped free of connective tissue ensheathment and cut into small pieces before being placed into collagen-coated Aclar wells (33C Aclar, gauge 5, Allied Chemical Co., Morristown, NJ; cf. reference 5). The neurons were grown for 3 to 4 d in vitro in Leibovitz's medium (L15; Gibco Laboratories, Grand Island, NY) containing 10% human placental serum, 9 mg/ml glucose, 2.5S nerve growth factor prepared from male mouse submaxillary glands (calibrated to produce maximal outgrowth in these neurons), and mitotic inhibitors (cytosine-1-β-D-arabinofuranoside and 5-fluorodeoxyuridine, both 10^{-5} M, with 10^{-5} M uridine added; all from Sigma Chemical Co., St. Louis, MO), but no antibiotics. The mitotic inhibitors effectively blocked proliferation of supporting cells of the ganglia. After 3–4 d in culture, at the time of the experiment, all explants were characterized by a broad halo of vigorous neuritic outgrowth (Fig. 1*A*). The use of the phosphate-buffered L15 medium, which is well liked by superior cervical ganglion neurons, has the advantage of permitting manipulation of the cultures under atmospheric conditions without drifting of the pH. For biochemical studies, we usually used, for each point, groups of five Aclar wells, each containing six explants. Groups of collagen-coated wells devoid of tissue served as blanks.

Pulse-chase Experiments: For pulse-chase experiments, the cultures were transferred into a 36°C constant-temperature room. They were pulsed for

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15 min with 2-[^{3}H]glycerol (0.5 mCi/ml; 9.5 Ci/mmol; New England Nuclear, Boston, MA), rinsed twice with medium containing 1 mM glycerol (corresponding approximately to a 20-fold excess of the cold precursor) and then incubated for different periods of time in the presence of fresh medium containing the cold precursor.

Biochemical Analyses: Immediately after the pulse, or following various chase periods, cultures were placed on ice and rinsed once more with ice-cold medium composed of L15, 1% bovine serum albumin (fraction V), and 1 mM glycerol. Still on ice, under a dissecting microscope, explants were excised from the cultures, carefully leaving intact and attached to the collagen the halo of neuritic outgrowth (Fig. 1; cf. reference 6). Explants were collected on small Millipore filters, washed once more with the same medium, and then dried; the Aclar wells containing the neuritic outgrowth were drained and then left to dry. After addition of 0.1 ml of water, both preparations were extracted with 1.9 ml of chloroform-methanol (2:1) overnight, according to the method of Folch et al. (9). After addition of methanol and water, the upper phase was discarded and the lower phase partitioned once more against fresh "upper-phase fluid" in order to remove unincorporated glycerol from the lipid phase as completely as possible. The lipid phase was then aliquotted out for inorganic phosphate analysis (in duplicate) and liquid scintillation counting. Inorganic phosphate was determined by a scaled-down version of the method of Ames and Dubin (2), which enabled us to measure reproducibly as little as 0.5 nmole of phosphate or 390 μg of phospholipid. The microgram amounts of phospholipid were determined by multiplying the amount of inorganic phosphate (in micromoles) by a factor of 775.

Net values for radioactivity and phospholipid were calculated by subtracting from the gross measurements for the cultures the respective values for collagen-coated, tissue-free wells run in parallel. Then the incorporation of [^{3}H]glycerol into phospholipid was expressed as specific radioactivity of phospholipid, i.e., as disintegrations per minute per microgram (dpm/μg)[1] phospholipid in the organic phase of the chloroform-methanol extracts. Because of considerable variation (*a*) in the number of neurons from culture to culture dish and (*b*) in the amounts of outgrowth and contamination by supporting cells between experimental series, the results were normalized as follows: the perikaryal values for the different time points in each set of experiments were expressed as percent of the highest perikaryal specific radioactivity found in each set. This was also done for the neurites, using the highest perikaryal value as a bias. This compensated for differences in the number of explants and neurons included in each sample of a series of time points. The resulting percent values for neurites in each set of experiments were normalized once more so that the highest value was 100%. This was necessary to adjust for differences in the amount of outgrowth obtained in the different series of experiments. The normalized percent figures for perikarya and neurites obtained for corresponding time points in the various experiment series were then averaged. Finally, these means were converted into dpm/μg phospholipid, using as a bias the actual average specific radioactivities at 30 and 90 min (highest values supported by as many as or more than four measurements) for perikarya and neurites, respectively. For the single experiment with 2,4-dinitrophenol (DNP), actual specific radioactivities are indicated.

Thin-layer chromatography of chloroform-methanol extracts was performed by spotting the concentrated samples on silica-impregnated glass fiber strips (Instant Thin-Layer Chromatography type SG, Gelman Instrument Co., Ann Arbor, MI) and running them first for 11 cm with chloroform-methanol-water (60:30:4). After drying, the chromatograms were developed once more, in the same direction, with 1% sodium borate in water, past the former solvent front to the 16-cm mark (19). The strips were then dried and cut into 1-cm segments for counting in a liquid scintillation spectrometer.

RESULTS

Incorporation of [^{3}H]Glycerol

To study the metabolic fate of 2-[^{3}H]glycerol in primary nerve tissue cultures, we exposed such cultures for 2 h to 0.5 mCi/ml of the precursor, then rinsed with chase medium and, finally, extracted with chloroform-methanol as described. As shown in Table I, >90% of the radioactivity appears in the organic phase and only a negligibly small fraction in the pellet, which is composed mainly of protein. The result of the subfractionation of the organic phase by thin-layer chromatography is even more important. As shown in Fig. 2, 97.3% of the total radioactivity loaded on the instant thin-layer chromatography strip migrates as phospholipid, whereas no significant counts are found at the origin or at the sodium borate front, which contains compounds soluble in an aqueous environment. Thus, the analysis of the organic phases of chloroform-methanol extracts in this system yields reliable data on the incorporation of 2-[^{3}H]glycerol into phospholipid.

[1] *Abbreviations used in this paper:* DNP, 2,4-dinitrophenol; dpm, disintegrations per minute.

TABLE I

Chloroform-Methanol Extraction of Cultures Labeled with [^{3}H]-Glycerol

	Net cpm*	% of total extract
Aqueous phase	5,810	7.0
Organic phase		
Total	76,720	92.5
Phospholipid	74,650‡	90.0
Pellet (washed)	430	0.52
Total extract	82,960	100.0

* Counts in sample minus counts in tissue-free control processed in parallel; rounded to the nearest 10 cpm.

‡ Calculated from instant thin-layer chromatography result as 97.3% of total organic phase.

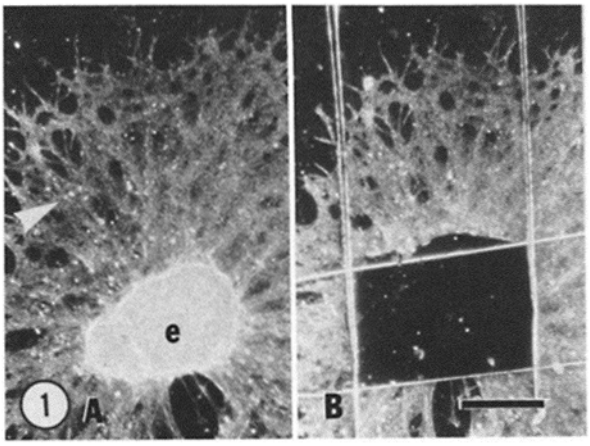

FIGURE 1 Explant culture of rat superior cervical ganglion, grown in vitro for 4 d, before (*A*) and after (*B*) microdissection of the explant (e) containing neuronal perikarya. Note that the peripheral outgrowth contains an extensive network of sprouting neurites. It is only very sparsely populated by supporting cells (arrowhead) that have emigrated from the explant. Bar, 100 μm. × 106.

Phospholipid Synthesis and Transfer into Neurites

Cultures of rat superior cervical ganglion were pulsed for 15 min with 0.5 mCi/ml and subsequently chased for various periods of time, up to 165 min. Perikaryal and neuritic fractions were then microsurgically separated and their phospholipid extracts prepared as described. An example of a culture is shown in Fig. 1*A* and, after microdissection of the explant, in Fig. 1*B*. The remaining neurite halo is largely, but not entirely, free of supporting cells that have emigrated from the explant into the periphery. The biochemical results of these pulse-chase experiments are shown in Fig. 3*A*, where the specific radioactivities of phospholipid for neurites and perikarya have been plotted as a function of time. As expected, there is a steep rise of specific radioactivity in the perikarya. However, this is followed by a rapid, exponential decline. The specific radioactivity of phospholipid is diluted fast, resulting

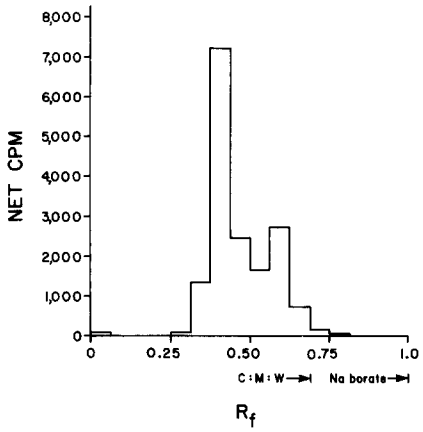

FIGURE 2 Thin-layer chromatography of chloroform-methanol extract of cultures labeled with 2-[^{3}H]glycerol (see Materials and Methods). 97.3% of the total radioactivity is in the phospholipid peak. Only minimal amounts are at the origin and the sodium borate front, respectively (cf. Table I).

in an apparent half-life of newly synthesized phospholipid of approximately $t_{1/2} = 90$ min.

The specific radioactivity of phospholipid in the neurites follows a time course that is entirely different from that in the perikarya. For the first 45 to 60 min following onset of the pulse, the specific radioactivity is at a constant, elevated level. Only after this lag time is there a gradual increase in specific radioactivity. This seems to level off somewhat, but has not yet reached a plateau, at 180 min. From the changes in specific radioactivity of the neurite and the perikaryon fractions and the respective phospholipid contents, it is possible to calculate the average total radioactivity transferred in the form of phospholipid from the perikarya to the neurites. The results of such calculations are shown in Table II. The phospholipid content of the neurite fraction (Table II, column *a*) is nearly twice that of the perikarya and widely variable. The average decrease in total radioactivity of perikarya within a 2-h period is ~58,000 dpm. The average increase of radioactivity in the neurites was found to be ~48,000 dpm. This value amounts to 83% of the perikaryal decrease. The difference can be explained by the incomplete recovery of the neurite fraction. Within the limits of these experiments, the values in Table II can therefore be considered to balance.

Influence of Blockers of Axoplasmic Transport

COLCHICINE: The experimentation was identical to the one just described, except that the cultures were treated for 30 min before and during the pulse-chase procedures with 2.5 or 5 × 10^{-6} M colchicine (the results for the two colchicine concentrations were not significantly different from one another). The findings for four sets of experiments with colchicine are shown in Fig. 3*B*. As in the normal controls (Fig. 3*A*), perikaryal specific radioactivity reaches a peak by 30 min following onset of the pulse, and that peak is at essentially the same level as in the controls. However, the subsequent dramatic decline in specific radioactivity observed in the control experiments is not evident after colchicine treatment. The fitted curve (assuming, as in the controls, an exponential decrease from 30 min on) indicates a decrease of only 14% during the time required for a 50% drop in the controls.

The specific radioactivity of phospholipid in the neurite fraction starts at a level similar to that of controls but fails to increase after 60 min following onset of the experiments. Thus, there is no evidence for the transfer of significant amounts of radioactivity from the perikarya into the neurite fraction.

2,4-DINITROPHENOL: In a further set of experiments, we used the metabolic blocker DNP to block all active processes in the cells and, therefore, any form of active transport of newly synthesized phospholipid from the perikarya into neuritic outgrowth. During the chase period, 5 min after the pulse with 2-[^{3}H]glycerol, these cultures were treated with 10^{-3} M DNP in chase medium. The results of these experiments are shown in Fig. 3*C*. This graph represents data points from a single experiment series. Although the specific radioactivity reached by the perikarya is not so high as for the previous experiments, the effect of the treatment with DNP is quite similar to that observed after colchicine in that the

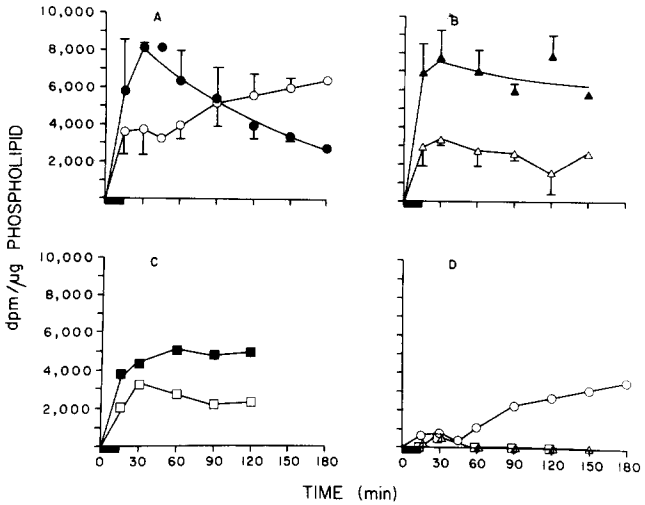

FIGURE 3 Specific radioactivity of phospholipid in explants (perikarya, solid symbols) and outgrowth (neurites, open symbols), plotted as a function of time following onset of a 15-min pulse with 2-[^{3}H]glycerol. The values indicated are normalized means plus-or-minus standard deviations, calculated as described in Materials and Methods. (*A*) Normal control. The curve for the decline in perikaryal values was obtained by fitting the points starting at 30 min with an exponential function ($r^2 = 0.98$). (*B*) Cultures treated with 2.5 to 5 × 10^{-6} M colchicine 30 min before, and during, the experiment. To conform with the data representation for controls, perikaryal values (solid triangles) for 30 to 150 min were also fitted with an exponential function. However, the number of experimental values for each point was smaller than for controls so that there is greater scatter of the means. (*C*) Cultures treated with the uncoupler DNP 5 min following the pulse with [^{3}H]glycerol. Results from a single experiment. Note that there is no significant transfer of radioactivity from perikarya into the neurites in colchicine- or DNP-treated cultures. In all three experiments, neuritic values are elevated to ~3,000 dpm/μg phospholipid as early as 15 min following onset of the pulse. This is most likely due to contamination of this fraction with supporting cells (cf. Discussion). In *D*, this base-level of radioactivity (average of all 15-min points in all experiments) has been subtracted from neuritic values for more direct comparison of time-dependent changes in drug-treated (open triangles, colchicine; open squares, DNP) and control neurites (open circles). Error bars have been omitted for the sake of clarity. From 60 min on, there is a statistically significant increase in control values (cf. Table III). For further description of results, see text, and for a listing of the number of data points, see Table III.

TABLE II

Transfer of Newly Synthesized Phospholipid from Perikarya into Distal Neurites

	a μg phospholipid	b 30 min	c 150 min	d
Perikarya	12.5 ± 1.0			
dpm/μg phospholipid*		8,100	3,470	
Total phospholipid dpm‡		101,300	43,380	−57,920
Neurites	21.6 ± 3.6			
dpm/μg phospholipid*		3,730	5,970	
Total phospholipid dpm‡		80,650	128,970	+48,320

(a) Average phospholipid content ±SEM (n = 52) per group of explants or neurite halos. Note that explants contain some supporting cells, and that the losses of phospholipid from the neurite fraction are considerably greater than those from the perikaryal fraction. (*b* and *c*) Radioactivities at time from onset of pulse with [^{3}H]glycerol. (*d*) Difference between *b* and *c*, i.e., radioactivity lost or gained during 120 min.

* Specific radioactivity of phospholipid.

‡ Total phospholipid counts, rounded to nearest 10 dpm.

TABLE III

Specific Radioactivities of Control and Colchicine-treated Neurites: Statistical Analysis

Time after onset of pulse	dpm/μg PL* control (n)	dpm/μg PL* colchicine (n)	$P<$
(min)			
15	3,580 ± 1,200 (4)	2,920 ± 1,010 (3)	ns
30	3,730 ± 1,360 (6)	3,320 ± 280 (4)	ns
60	3,960 ± 660 (6)	2,710 ± 870 (3)	0.05
90	5,160 ± 1,250 (6)	2,590 ± 320 (3)	0.015
120	5,590 ± 1,240 (4)	1,590 ± 1,250 (3)	0.01
150	5,970 ± 640 (2)	2,520 (1)	

n, number of data points; p, significance of the difference between treated and control cultures, determined by Student's t test. *ns*, not significant.

* Specific radioactivity of phospholipid, rounded to nearest 10 dpm, ± standard deviation.

rapid decline of radioactivity seen after 30–45 min in the control does not occur. Analogously, there is no increase in the specific radioactivity of phospholipid of the neurite fraction.

If we assume that the initial level of radioactivity in phospholipid of the neurite fraction, ~3,000 dpm/μg, constitutes a background contributed by supporting-cell contamination of this fraction, then we can substract this radioactivity from all neuritic values and re-plot the time course of specific radioactivity for the three types of experiments described above. This has been done in Fig. 3*D*. For clarity, the standard deviations have been omitted from this figure. The differences between controls and experiments with colchicine or DNP are now dramatically evident. These differences have also been analyzed statistically by Student's t test. As shown in Table III, the divergence of specific radioactivities of phospholipid in the neurite fraction is statistically significant at and after 60 min following onset of the experiment.

DISCUSSION

Biochemical studies on primary neurons grown in culture are somewhat difficult because of the very small amounts of material available, because of the degree of variation in the amount of outgrowth produced, and because of the very fragile nature of the neurites. However, this experimental system offers two major advantages: the transfer of materials from one part of the cell to another can be analyzed quantitatively by separating the two portions microsurgically, and true pulse-chase experiments can be carried out (which would not be possible by injection of a radioisotope in vivo). Furthermore, the chosen precursor for phospholipid, 2-[^{3}H]glycerol (cf. reference 3), is particularly suitable in this system because virtually all of the incorporated radioactivity is found in the desired family of substances. Thus, it is possible to investigate whether newly synthesized phospholipid is distributed in the sprouting neuron at random or whether it is preferentially transported into the growing neurites.

Selective Transfer of Newly Synthesized Phospholipid into the Growing Neurite

The rapid and massive accumulation of radioactivity in the neuronal perikarya marks them as the major sites of phospholipid synthesis. Yet, the biochemical results cannot exclude the possibility of incorporation into phospholipid within the neurites. This possibility is unlikely, though, for the following reason: during the first 15 min after the [^{3}H]glycerol pulse, when perikaryal values continue to increase substantially, there is no significant increase in neuritic values in control and colchicine experiments. Therefore, if it is assumed that most—if not all—of the radioactivity detected biochemically in the neurite fractions at the earliest time points is derived from contaminating supporting cells, neuritic phospholipid synthesis occurs at a very slow rate or not at all. The perikaryon turns out to be a transient site of newly synthesized phospholipid. Well over one half (57%, cf. Table II) of phospholipid synthesized in the first 30 min of the experiment leaves the perikarya within the next 2 h. Our calculations indicate essentially quantitative transfer of this phospholipid into the sprouting neurites.

The increase in specific radioactivity of the neurites, as determined biochemically, is delayed by nearly 60 min and then seems to proceed, approximately mirroring the decline in perikaryal values. However, the increase in specific radioactivity of the neurites is not as dramatic as its decrease in the perikarya. This is easily explained by the fact that newly synthesized phospholipid gets diluted in the neurites in a pool of phospholipid that is nearly twice as large as that of the perikaryal fraction. In summary, the results of this study enable us to conclude that the majority of newly synthesized phospholipid in the sprouting neuron is rapidly transferred into the neuritic outgrowth.

Mode of Transfer of Newly Synthesized Phospholipid

The rate of transport of newly synthesized phospholipid into the growing neurites can only be estimated from these studies. The outgrowth halo of the average explant in this study has a total diameter of ~4.5 mm. The explant has an average diameter of ~0.2 mm. This means that radioactive phospholipid has to travel ~2.2 mm before reaching the nerve growth cones. If we assume that the first significant amounts of radioactive phospholipid appear in the growth cones 30 min after onset of the experiment (as the radioau-

tographic analysis indicates; data to be presented in a forthcoming paper), and that uptake and incorporation of the label require 10 min, this results in a rate of ~2.2 mm/20 min or 160 mm/d. This estimated transport rate falls well into the range of rapid axoplasmic transport (e.g., references 10, 20). This conclusion is consistent with results of the experiments using DNP or colchicine. The uncoupling of cellular metabolism by DNP is certain to block active transport in the cell and results in the inhibition of transfer of newly synthesized phospholipid from the perikarya into the neurites. Thus, there is no evidence for proximo-distal diffusion (e.g., laterally within the plasma membrane) of phospholipid synthesized and inserted into perikaryal plasmalemma during the 20 min preceding DNP treatment. The drug colchicine is considered to be a rather selective inhibitor of rapid axoplasmic transport (for review, see references 10, 20). Indeed, colchicine treatment prevents the transfer of newly synthesized phospholipid into the neurites. Therefore, we can infer that the newly synthesized phospholipids travel by rapid axoplasmic transport from the perikaryon into the distal parts of the neuron. This conclusion implies that the phospholipids are transported in formed elements such as vesicular structures because all substances found to date that travel at the rapid rate are particulate in nature. Such an interpretation of the results is consistent with what is known about the export of membrane components, particularly phospholipids, into the axon in the mature neuron (1, 4, 11, 13; for review, see references 10, 20) as well as with current views of membrane biogenesis in general (see reference 8 and, for review, see references 7, 12, 14, 18).

In summary, our results indicate that the perikaryon is the growing neuron's major—if not exclusive—site of phospholipid synthesis. Furthermore, the growing neuron transports the membrane lipids necessary for plasmalemmal expansion specifically into the growing neurite, most likely in the form of preassembled, membranous structures such as vesicles. Therefore, our results are complementary to those obtained by pulse-chase experiments with surface labels (17), which indicated localized insertion of new membrane glycoconjugates at the level of the nerve growth cone. A third, complementary and independent line of evidence comes from freeze-fracture studies, which tentatively identify, on the basis of the respective intramembrane particle contents, a class of large vesicles as the plasmalemmal precursor (16; R. K. Small and K. H. Pfenninger, manuscript in preparation). The identification of the plasmalemmal precursor by high-resolution radioautography will be the topic of a forthcoming communication (K. H. Pfenninger and L. B. Friedman, manuscript in preparation). However, we can already conclude that certain cells, such as the sprouting neuron, expand their plasmalemma by vectorial export of certain membrane components, including phospholipids, and by localized insertion of these components at or near the growing tip.

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