Plasma membrane expansion: a neuron's Herculean task

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Abstract | The formation of axons and dendrites and maintenance of the neuron's vastly expanded surface require the continuous addition of new membrane. This is achieved by membrane synthesis through the secretory pathway followed by regulated vesicle fusion with the plasma membrane, typically in the distal neurite. However, it is far from simple: multiple distinct membrane carriers are used to target specific membrane domains, dendrites seem to operate semi-autonomously from the rest of the neuron, and exocytosis for membrane expansion is different from that for release of synaptic vesicles. Current knowledge of this process and its implications for neuronal development, function and repair are reviewed.

Growth cone
The terminally enlarged, amoeboid tip of a neurite.

Perikaryon
The portion of the neuron that surrounds the nucleus and excludes the neurites. Also referred to as the cell body.

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This Review is dedicated to George E. Palade, the father of the secretory pathway, who passed away on 7 October 2008.

The plasma membrane (or plasmalemma) enables neurons to generate and propagate ionic currents, two of their most important cell type-specific functions. It is the neuron's largest organelle, with a typical surface area of ~250,000 μm^2. This area can easily reach into the millions of square micrometres for neurons with long axons[1,2]. To put this in perspective, the surface of a spherical cell with a diameter of 20 μm amounts to only 1,256 μm^2. Generating and maintaining the neuron's huge surface area is further complicated by the presence of distinct membrane domains in both development and maturity. This has to be taken into account when considering the mechanisms and locations of plasmalemmal synthesis, assembly and expansion.

There are at least four different conditions of neurite growth and plasmalemmal expansion *in vivo*: first, during *de novo* outgrowth of a neurite tipped by a growth cone (FIGS 1a,2a); second, during the continued elongation of axons concomitant with growth of the animal — this occurs once the axon has reached its target and formed a synapse (growth of networked processes (FIG. 1b)); third, during the formation of collateral sprouts (in the developing and mature nervous systems (FIG. 1c)); and fourth, during the regeneration of a severed neurite.

A neurite of 1 μm diameter elongating at 0.5 mm per day — fairly typical numbers for a mammalian neuron — must expand its surface at a rate of ~1 μm^2 per min. Moreover, the surface area/volume ratio of the growing neuron increases dramatically with neurite extension. Using the example of a 1 μm-diameter axon elongating at a rate of 0.5 mm per day and assuming a perikaryon of 50 μm diameter, the volume of the cell increases by only 0.6% per day, whereas the surface (that is, the plasmalemma) increases by 20% per day.

Hughes was one of the first to report, back in 1953, on mechanisms of neurite growth. He found that the distal segment of a severed neurite continued to grow for some hours, suggesting the presence of a distal reservoir of membrane components[3]. Membrane growth can occur remote from the perikaryon, where most of the membrane-synthetic machinery is located. Thus, neuronal membrane expansion is unique in terms of its huge scale, the long distance between sites of synthesis and neurite growth, and the emergence of distinct membrane domains. Evidently, membrane expansion is a complex and crucial aspect of neurite growth. As such, it is likely to have a role in disease processes affecting nerve growth, maintenance and regeneration.

This Review discusses our understanding of membrane expansion in polarized vertebrate neurons, raising some new and unanswered questions. The data come from both central and peripheral neurons. However, membrane expansion processes may not be identical in the two systems, and generalizations to neurons of different geometry in vertebrates or invertebrates should be made with caution.

Membrane synthesis and transport in the neuron

The neuron's perikaryon is the primary site of synthesis of large molecules. Rough endoplasmic reticulum (ER) and Golgi complexes, in which membrane proteins are synthesized and modified, are abundant in the

Microtubule plus end
Microtubules are polarized structures, and the plus end is the end at which polymerization predominantly occurs. By contrast, disassembly predominates at the minus end. In axons, microtubules are oriented so that the plus ends point towards the growth cone or presynaptic terminal.

perikaryon and extend into large dendrites (BOX 1). The axon hillock prevents these organelles from entering the axon through a barrier mechanism that involves the microtubule motor dynein[4]. However, many vesicular structures, smooth membrane sacs and mitochondria can pass through. Thus, the bulk of protein and lipid synthesis, and of glycosylation and other Golgi-based processing steps, occurs in the perikaryon. Numerous studies on the incorporation of radio-labelled amino acids and glycerol into protein and phospholipid, respectively, and on their subsequent axoplasmic transport confirm this concept. Studies on the synthesis and export of model cargo proteins and of fluorescent fusion proteins and lipids further establish that, in terms of membrane biogenesis, the neuronal perikaryon generates one or multiple vesicular compartments destined for export into the axon and dendrites (BOX 2).

Plasmalemmal precursor vesicles (PPVs). Neurons are known to synthesize various vesicular structures, including synaptic and large dense-core vesicles. Identifying the vesicles that are destined for plasmalemmal expansion, the PPVs, has been problematic. Clusters of relatively large (~150 nm diameter) pleomorphic vesicles were documented in the earliest electron micrographs of nerve growth cones[5]. Later studies showed that they were grouped at sites of insertion of glycoconjugates into the plasmalemma, and that they were enriched in newly synthesized phospholipid[6]. Finally, membrane-expansion assays performed on isolated growth cones demonstrated a decrease in these vesicles in conjunction with plasmalemmal expansion[7]. Vesicles with PPV morphology have been observed in growing axons and dendrites[8,9] as well as regenerating axons[10], but they are quite rare in adult nerve tissue. Interestingly, similar vesicles are also found in clusters adjacent to the cleavage furrow in cells undergoing cytokinesis, during cellularization of the *Drosophila melanogaster* embryo and at the tips of hyphae and pollen tubes (BOX 2).

These findings identify PPVs of the growing neuron only ultrastructurally. Owing to the lack of specific biochemical markers, functional experiments that link PPVs to plasmalemmal expansion have been carried out using pulse-labelling strategies to identify the most recently synthesized vesicles. These approaches do not distinguish the different types of PPV that have been reported[11–14]. It seems that at least three different PPV types carrying diverse sets of membrane proteins are transported in the axon by different microtubule plus end-directed vesicle motors of the kinesin family: KIF2, KIF4 and kinesin[11–14]. Implicit to these observations is the conclusion that the primary transport mechanism of PPVs is microtubule-based. This is consistent with the fact that microtubule toxins block axonal growth whereas actin-depolymerizing drugs do not[15].

Distal protein and lipid synthesis. The transport of mRNAs into axons and dendrites and the presence of polyribosomes and rough ER in larger dendrites suggest that axons and dendrites are sites of protein synthesis. Numerous studies have provided evidence that this is the case, at least during outgrowth[16–19].

Proteins synthesized in axonal growth cones seem to be primarily of regulatory and cytoskeletal nature (they are synthesized on free ribosomes) and have been shown to affect growth cone turning and advance as well as growth cone formation after axotomy[19,20]. However, at least two transmembrane proteins with established or consensus glycosylation sites — the κ-opioid receptor and the guidance receptor EPHA2 — are also axonally synthesized[21,22], even though there is no evidence for the presence of rough ER or Golgi-like elements. This conundrum necessitates a thorough search for appropriate structures and biochemical markers in unambiguously identified differentiated axons and their growth cones.

The question of distal phospholipid synthesis was raised by the discovery of high arachidonic acid release and turnover in axonal growth cones[23]. However, incorporation of the phospholipid backbone, glycerol, occurs predominantly at the perikaryon, and is followed by *quasi*-quantitative transfer of newly synthesized phospholipid into the growing axon[24]. Therefore, it is likely that fatty-acid release and reincorporation into phospholipid reflects primarily lipid remodelling rather than *de novo* synthesis[23].

In summary, protein synthesis and lipid remodelling (and perhaps limited synthesis) do occur in the axon. Although functionally important, these processes are quantitatively minor contributors to membrane biogenesis[25]. The bulk of this process occurs in the perikaryon,

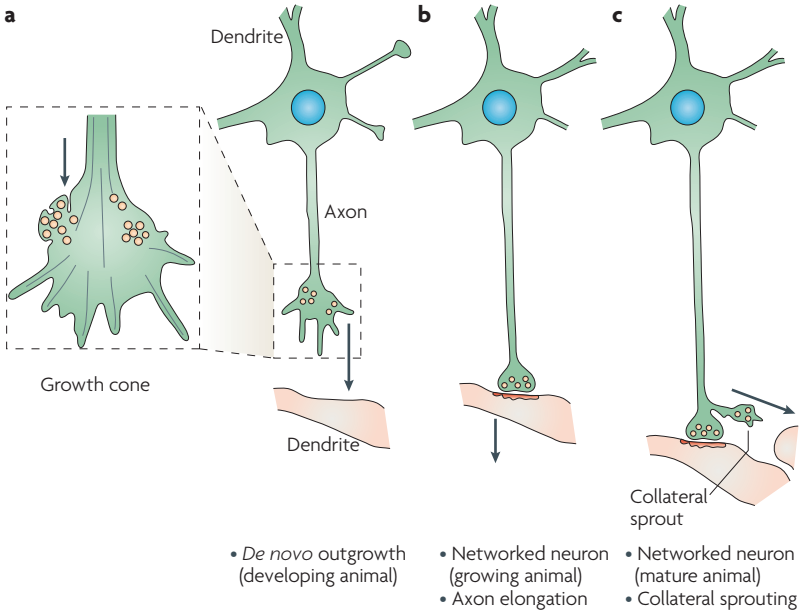


Figure 1 | **Modes of axonal growth *in vivo*. a** | During early development, neurite elongation towards a target dendrite occurs through *de novo* outgrowth of processes tipped by growth cones. Plasmalemmal precursor vesicles clustered against the plasmalemma are shown in the inset. These are sites of membrane insertion and are also characterized by plasmalemmal invaginations of unknown function (indicated by the arrow). Dendrites and regenerating axons have similar characteristics. **b** | Growth of a networked, synapsing axon. As the animal grows, networked axons must continue to increase in length. This process is poorly understood. **c** | Formation of an axonal collateral sprout in the mature nervous system. Collateral sprouting seems to follow the principles of neurite outgrowth, involving a growth cone-like structure.

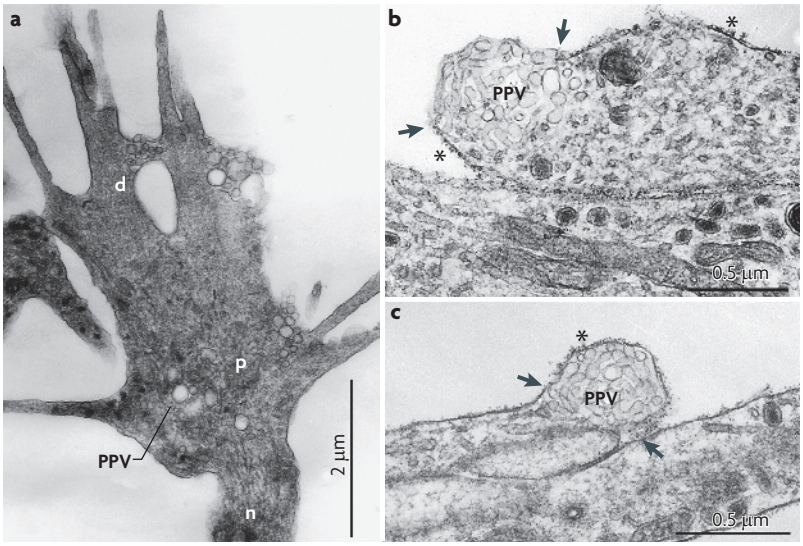


Figure 2 | **Plasmalemmal precursor vesicles (PPVs) and glycoconjugate externalization in the growth cone. a** | An electron micrograph of a growth cone (from a rat spinal cord neuron) in culture, showing proximal (p) and distal (d) domains. Several clusters of PPVs can be identified. **b**,**c** | Pulse–chase experiments with lectins (wheat germ agglutinin) and lectin–ferritin conjugates show PPV clusters associated with growth cones in culture. After a ferritin–lectin pulse and chase for 15 min in the absence of the label, the plasmalemma covering the vesicle cluster is essentially label-free (**b**). If the labelling pulse is carried out with unconjugated lectin, followed by ferritin–lectin label after the chase period, the PPV clusters are covered with ferritin particles (**c**). Asterisks indicate areas of high ferritin label density; arrowheads approximately mark the transition from label-rich to label-poor plasmalemma. These results demonstrate insertion of new glycoconjugates into the plasmalemma at PPV clusters. n, neurite. Part **a** is reproduced, with permission, from REF. 127 © (1976) Plenum Press. Parts **b** and **c** are reproduced, with permission, from REF. 41 © (1981) Rockefeller University Press.

following the steps of the secretory pathway. After budding from the *trans*-Golgi network (TGN), PPVs are transported into the growing neurites (BOX 2; FIG. 3).

Alternate pathways of membrane transport. The growing neuron exhibits distinct plasmalemmal domains[26–28]. The enrichment of certain molecular species in specific membrane domains can be accomplished, in principle, in three different ways: through the sorting of components in the TGN and the formation of distinct vesicle types that are targeted to select regions (an example is the axonal growth cone's insulin-like growth factor 1 (IGF1) receptor (IGF1R)[11]); through omni-directional export of components followed by selective retrieval and degradation of specific components in some regions but not others (this has been shown for a voltage-gated Na^+ channel and for the vesicle SNARE (SNAP receptor) vesicle-associated membrane protein 2 (VAMP2)[29,30] (BOX 3)); and through transcytosis — that is, targeting to one domain followed by endocytosis (without degradation) and shuttling to another region, a process that is well established for apical proteins in epithelial cells[31] and for the cell-adhesion molecule L1 (also known as NRCAM and, in chick, NGCAM) in hippocampal neurons[32]. Appropriate export depends on targeting sequences in the cargo proteins[32]. All three mechanisms are active in growing neurons (FIG. 3), but their relative roles change as the neuron differentiates. For example, whereas omni-directional export predominates at first, more specific targeting later ensues[33].

In epithelial cells distinct plasmalemmal domains are kept separate by the formation of junctional belts, including tight junctions[34]. In neurons the axon hillock has this role to some extent, but it does not seem to be fully functional during neurite outgrowth. Freeze-fracture studies suggest that some equilibration between compartments may occur through lateral diffusion of components in the membrane. These analyses demonstrate that the perikaryon contains a high density of intramembrane particles (IMPs) whereas the growth cone's plasmalemma is IMP-poor, and that IMP density has a proximo-distal gradient that can be modelled by diffusion in a moving-boundary system[35]. The molecular identities of these IMPs are not known, but the results indicate that such proximo-distal diffusion may be a further mode of transfer of membrane components into neurites.

Regulated membrane insertion

Secretory-vesicle exocytosis may be constitutive or regulated. In the latter case, secretory vesicles accumulate at potential sites of exocytosis but membrane fusion and content release must be triggered by a stimulus. Initially it was assumed that addition of membrane components to the plasmalemma was a constitutive phenomenon (for example, see REF. 36). Although this may be the case for certain cell lines, including fibroblasts, there is substantial evidence for a regulated mechanism in neurons[37]. Another important issue is where in the growing neuron the newly synthesized membrane is inserted — proximally at the perikaryon, distally at the growth cone or randomly. The clustering of PPV-like vesicles at specific growth cone locations suggests the presence of distal sites of regulated exocytosis and membrane insertion[6].

Growth cones exhibit three distinct regions (FIG. 2a): a peripheral zone characterized by actin-rich, thin lamellipodia and filopodia containing few membranous organelles; a proximal or central zone that is rich in organelles including mitochondria and various vesicular structures; and an intermediate, transitional zone where microtubules dominate over microfilaments. In growth cones in culture, this transitional zone often contains large, clear, coat-free vesicles clustered against the plasmalemma. Interestingly, such clusters are surrounded by a series of plasmalemmal invaginations that extend as a network underneath the vesicle clusters and can be filled with extracellular markers[5,6] (FIG. 1a). The function of these invaginations is unknown, but the vesicles have the morphological characteristics of PPVs.

From vesicle to plasma membrane. By using diverse membrane labels, various studies have shown that membrane components move from PPV-like vesicles to the cell surface[6,38–41].

When live growth cones were labelled with ferritin-conjugated lectins and then incubated in the absence of label, they exhibited label-free membrane patches above clusters of pleomorphic 150 nm diameter vesicles and extending to the growth cone periphery (FIG. 2b). In the

Trans-Golgi network
The last stage of the Golgi complex, where sorting of components and vesicle formation take place.

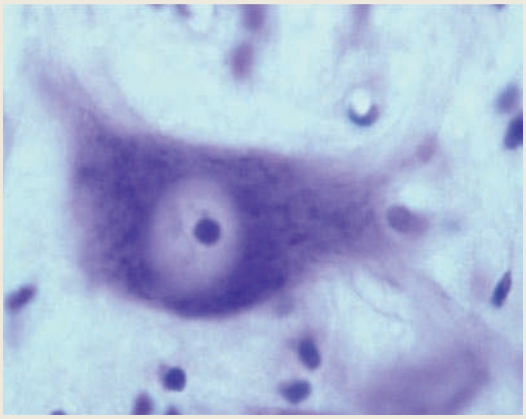
Freeze fracture
A preparative method for electron microscopy that involves rapidly freezing and fracturing the biological sample and then shadow casting it with a metal vapour to generate a replica of the fracture face. Ultrastructural analysis of the replica reveals structures in the lipid bilayer, such as intramembrane particles, that are thought to represent membrane-protein complexes.

Moving-boundary system
A system that expands with time so that diffusion in it never reaches equilibrium, as long as expansion (in this case neurite growth) exceeds the diffusion rate.

Lectin
A protein that recognizes and binds to specific oligosaccharide sequences.

Excimer
Very transient dimers of fluorescent molecules, one of which is in an excited state. Emission during return to the ground state is of lower energy (red shift) than that of excited monomers. The term is a contraction of 'excited dimer'.

Box 1 | **The mystery of the Nissl substance**



Approximately 100 years ago, the German neuropathologist Franz Nissl (1860–1919) discovered one of the neuron's most characteristic cytological features, the presence of masses of a granular material in the perikaryon and large dendrites. The basic dye he used to reveal the 'Nissl substance' was later found to be selective for RNA. The Nissl substance thus represents large stacks of rough endoplasmic reticulum. At the time of discovery the abundance of this organelle was a complete enigma for a cell that does not secrete large quantities of protein (such as cells of the liver or the exocrine pancreas). It was decades before the neuron's characteristic and abundant translational machinery was linked to the synthesis of its membrane proteins. The figure is a light micrograph of a motor neuron in the cat spinal cord stained using Nissl's technique.

inverse experiment, live growth cones were pulse-labelled with unconjugated (invisible) lectin to block exposed glycoconjugates, later fixed and finally labelled with ferritin-conjugated lectin (FIG. 2c). Such growth cones exhibited above the vesicle clusters (and occasionally continuing into the lumen of the vesicles) patches of membrane label that spread to the growth cone periphery[6,41]. Thus, the appearance of label-free areas in the first experiment was not simply the result of label redistribution; it was caused by the insertion of glycoconjugates from an internal compartment (inccessible to the label) and the spreading of these glycoconjugates to the distal growth cone.

These studies identified morphologically distinct sites of vesicle clustering in growth cones, where transfer of membrane glycoconjugates from PPVs to the plasmalemma occurs[38–40], and validate Bray's suggestion in the 1970s of distal membrane expansion[42].

Box 2 | **General principles of membrane expansion**

Owing to the amphiphilic nature of phospholipids and the aggregates they form, membranes grow from pre-existing membranes by the insertion of new components. Lipid synthesis occurs in membranes of the endoplasmic reticulum (ER), and the membrane's compositional lipid asymmetry — with aminophospholipids, such as phosphatidyl ethanolamine, in the cytoplasmic leaflet and phosphatidylcholine and sphingomyelin enriched only in the exoplasmic leaflet — is established here and/or in the *trans*-Golgi network by ATP-dependent lipid flippases[65,115]. Integral membrane proteins are typically incorporated co-translationally in the rough ER. However, proteins anchored to the membrane through their carboxyl terminus, such as the vesicle-associated membrane proteins (VAMPs) and the syntaxins (BOX 3), are post-translationally inserted[116]. Through vesicular transport, new membrane reaches the Golgi complex for further processing and sorting and, subsequently, the plasma membrane for insertion. The underlying vesicle fission and fusion processes maintain compartment integrity and membrane continuity.

This process, known as the secretory pathway[117–119], transfers plasmalemmal precursor vesicles (PPVs) to the cell surface. Although insertion of PPVs might occur at random in non-polarized cells, it is known to be specifically targeted in a number of non-neuronal cells, such as: polarized epithelial cells, in which different membrane components are shipped selectively to either the basolateral or the apical plasma membrane[120,121]; cells undergoing cytokinesis, in which PPVs fuse with the plasmalemma primarily in the cleavage furrow[122]; cells in *Drosophila melanogaster* embryos during cellularization, in which membrane addition takes place at the emerging intercellular septa[123]; and cells in growing hyphae (root hairs) and pollen tubes, in which exocytotic membrane expansion occurs specifically at the tip[124]. These highly diverse eukaryotic examples illustrate the fact that new membrane is added specifically where it is needed. Selective targeting of new membrane in polarized cells evidently offers the advantages of enabling the generation and maintenance of distinct membrane domains and local control of the process of surface expansion — for example, at the growing tip.

Regulation of insertion. Neurite outgrowth is the product of a coordinated set of processes, including plasmalemmal expansion, cytoskeletal growth and remodelling, and growth cone locomotion. Numerous factors participate in the regulation of these processes, and the intracellular levels of second messengers such as Ca^{2+} and cyclic nucleotides greatly influence the resulting level and direction of neurite growth (for example, see REFS 43–45). The present discussion is focused on the regulation of PPV insertion.

The clustering of PPVs suggests that membrane expansion in the growth cone is a regulated phenomenon. This hypothesis was tested in cell-free assays, involving subcellular fractions of isolated growth cones (GCPs), as well as in intact growth cones in culture. In the GCP experiments, Ca^{2+} influx caused externalization of membrane glycoconjugates and a concomitant decrease in the size of the pool of pleomorphic vesicles, which strongly argued that these vesicles are PPVs[7]. This was supported in intact growth cones in culture using BODIPY-ceramide, a fluorescent lipid derivative that is incorporated into sphingomyelin and gangliosides, as a membrane marker. The emission spectrum of BODIPY-ceramide is concentration-dependent, with a peak at 515 nm (green) at low concentrations. At high concentrations, excimer formation shifts emission to 620 nm (red), and in pulse–chase experiments neurons exhibit bright-red fluorescent Golgi complexes and vesicles in transit to the growth cone. Subsequent insertion of the vesicles into the plasma membrane, with concomitant dilution of the label, results in a green fluorescent growth cone plasmalemma, illustrating the precursor–product relationship between the Golgi-derived vesicles and the plasmalemma (FIG. 4). This technique was used to show that IGF1, through the insulin receptor substrate–phosphoinositide 3-kinase (PI3K)–AKT pathway, has an important role in mobilizing internal membrane glycoconjugates to the cell surface and regulating membrane expansion at growth cones[11,46] (FIG. 5).

Vesicle docking and fusion and their sequelae. Exocytosis requires two intimately linked but separate molecular machines — those for vesicle tethering and for membrane fusion (BOX 3). In many cases of regulated exocytosis the exocyst complex is involved in the docking of vesicles at the site of exocytosis[47,48]. Elements of this octameric protein complex, such as SEC6, SEC8 and SEC10, have been implicated in neurite growth[49–51]. They have been identified at the growth cone and in clusters along the growing axon. However, ultrastructural data on the localization of exocyst complexes relative to the growth cone's PPV insertion sites are not yet available. PI3K activity seems to be a universal requirement for growth factor-regulated insertion of membrane proteins into the plasmalemma[46,52–54]. Recent studies in adipocytes link PI3K to the plasmalemmal recruitment of the exocyst component EXO70 through activation of the small G protein TC10 (also known as RHOQ)[55] (FIG. 5).

Although we have extensive knowledge of the components involved in synaptic-vesicle exocytosis, little is known about PPV fusion with the plasmalemma. The plasma-membrane target SNAREs the syntaxins and synaptosomal-associated protein, 25 kDa (SNAP25) are present in the growing axon; more importantly, axonal growth *in vitro* requires the function of the fusion-machinery proteins SNAP25, syntaxin 3, syntaxin 12, TI-VAMP (a vesicular or R-SNARE; also known as VAMP7) and SEC1/MUNC18-like proteins[50,56–62]. Although the apparently normal neurite outgrowth in *Snap25*-null mice[63] might suggest that the fusion machinery is not required for axon growth, it is likely that functional compensation by the closely related *Snap23* is responsible[64] and that exocytosis is required for plasmalemmal expansion (FIG. 5).

One of the puzzles of neuronal membrane expansion is a topological problem involving the lipid bilayer (FIG. 6). In the relatively small PPVs the two lipid monolayers that form the membrane differ by ~25% in area owing to the vesicles' curvature (assuming a spherical shape). As vesicles are inserted into the plasma membrane their curvature switches: the smaller, luminal, concave lipid leaflet of the vesicle merges with the slightly larger, convex outer leaflet of the plasma membrane. This results in a shortfall of ~30% in lipid monolayer area and requires the transfer of lipid from the inner to the outer leaflet. Such flip-flop can be accomplished by lipid flippases. The importance of this problem is illustrated by the fact that yeast cells lacking ATP-dependent flippases are deficient in endocytic-vesicle formation, the inverse topological change[65] (see also REF. 66). Over time and in a rapidly expanding membrane system, however, this transfer would change the lipid composition of the outer leaflet because the leaflet facing the cytoplasm consists primarily of phosphatidyl ethanolamine whereas the outer leaflet's main component is phosphatidylcholine. The enzymes that convert the former lipid into the latter, phospholipid methyltransferases, are indeed present in growth cones[67]. However, the precise mechanisms involved in this membrane bilayer adjustment, which must be very active in the growing neuron, remain enigmatic.

Endocytosis and recycling. Endocytosis is a widespread phenomenon that serves the cellular intake of large soluble molecules and/or the removal from the cell surface of membrane components. In axonal growth cones,

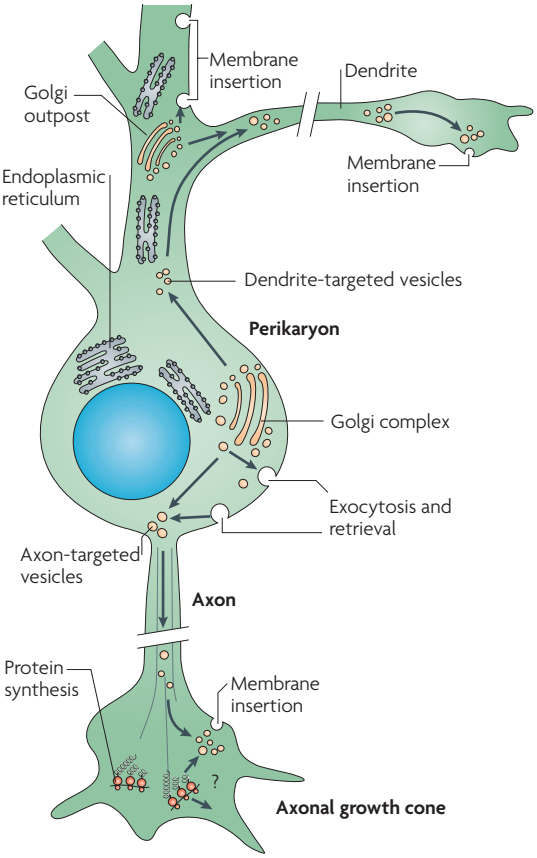


Figure 3 | **Sites of synthesis and plasmalemmal insertion of membrane components in a growing polarized neuron.** The perikaryon is the primary site of synthesis and generates plasmalemmal precursor vesicles (PPVs) for export into the axon and dendrites. During *de novo* axon outgrowth membrane expansion occurs primarily at the distal tip, the growth cone. Components of specifically targeted vesicles are pre-sorted in the *trans*-Golgi network. However, some axonal membrane proteins are inserted uniformly and retrieved by destructive endocytosis from domains where they do not belong. A third group of membrane proteins reaches the axon by transcytosis — that is, plasmalemmal insertion at the perikaryon and dendrites, followed by selective retrieval and targeting of endocytic vesicles to the axon. Protein synthesis occurs at the axon's growth cone, but it is unknown how locally synthesized membrane proteins are inserted into the plasma membrane. By contrast, dendrites contain all elements of the secretory pathway, including Golgi outposts, and generate their own PPVs in addition to those produced in the perikaryon. Such vesicles may be inserted at the growing tips of dendrites or along their shafts. Overall, the neuron uses the basic principles of the secretory pathway and primarily distal insertion to expand its plasma membrane. However, the process is not simple as there are different membrane domains to which the different types of PPV have to be appropriately targeted. Furthermore, membrane insertion is regulated independently in axons and dendrites.

ultrastructural studies with volume markers or cell-surface labels revealed the expected internalization of the tracer. However, label did not enter the PPV compartment, and so the endocytic and secretory pathways do not seem to intersect in the growth cone[6,41].

The immunoglobulin superfamily cell-adhesion molecules L1 and neural cell-adhesion molecule (NCAM) are recycled at easily detectable rates at the axonal growth cone. L1 recycling occurs locally in the central domain of growth cones advancing on a laminin matrix. However, when growth cones are grown on an L1 matrix, L1 is recycled to the growth cone's leading edge and then moves retrogradely on the cell surface to the central domain, suggesting that this process is related to the mechanism of amoeboid locomotion[68]. NCAM (complexed by antibody) is endocytosed at the growth cone by two different pathways, one of which is clathrin-dependent and the other is caveolae-dependent[69]. However, as for L1, most internalized NCAM is recycled to the cell surface rather than degraded in lysosomes. A potential third endocytic mechanism has been described more recently[70] and likened to macropinocytosis, but the putative large endosome compartment has not been characterized. Overall, although recycling of membrane components is an important growth cone phenomenon, net membrane retrieval and degradation seem to be minor components of membrane flux in the growing neurite.

Despite the gaps that evidently remain in our understanding of the mechanism of membrane insertion at the growth cone, we can draw a number of conclusions from the evidence summarized here: plasmalemmal expansion in the growing axon occurs primarily at the growth cone, it occurs through a mechanism of regulated exocytosis involving the exocyst complex and SNAREs, and it is regulated by growth factors. This mechanism is distinct from that of transmitter release[59] in that it uses different docking and fusion proteins.

Box 3 | **Exocytosis, the exocyst and the fusion machinery**

Exocytosis necessitates two distinct processes, vesicle docking or attachment and vesicle fusion[125,126]. The underlying mechanisms are mediated by separate protein complexes.

Rab proteins, activated by GTP binding, mediate vesicle attachment to the target plasma membrane. Mammals synthesize more than 60 different Rabs that can identify specific 'donor' membrane compartments in the cell. Such GTP-bound Rabs bind to specific 'effector molecules' on the target or 'acceptor' membrane to establish docking. One of the best-known effectors is the exocyst, which is often involved in transferring membrane components to the cell surface[47,48]. The exocyst is an octameric protein complex, first discovered in budding yeast but subsequently identified in various animal cells, that targets and docks vesicles to specific plasmalemmal insertion sites. Animal cell exocysts consist of the proteins SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70 and EXO84, and they interact with or are regulated by the small GTPases RAB11, TC10 (also known as RHOQ), RALA–RALB and ARF6. In polarized epithelial cells, exocysts are necessary for basolateral but not apical membrane targeting. In neurons, exocysts are involved in neurite outgrowth and synaptogenesis but not in synaptic transmitter release[49,126].

Whereas the Rab–effector complex is necessary for the assembly of the membrane-fusion machinery, there is no evidence for its direct involvement in the fusion mechanism. This is a function of the SNARE (SNAP receptor) and SM (SEC1/MUNC18-like) proteins[125,126]. The mammalian genome encodes more than 35 different SNAREs, which are membrane-associated (typically through a single transmembrane domain) and expressed selectively on specific membrane compartments. Fusion necessitates that four different SNARE protein subtypes form a complex. One of these, synaptobrevin (an R-SNARE), is contributed by the donor vesicle; the three others (Q-SNAREs, such as the syntaxins and synaptosomal-associated receptor, 25 kDa (SNAP25) homologues) are contributed by the target or acceptor membrane. Binding of a vesicular SNARE to the appropriate target SNAREs leads to the formation of the SNARE *trans* complex, which brings the membranes into close proximity for fusion. This complex seems to be regulated by the SM proteins, which are essential for the fusion event.

Axons versus dendrites

Neuronal differentiation entails many cellular changes, including neurite outgrowth and, eventually, polarization — that is, the emergence of an axon and dendrites. Hippocampal neurons in culture initially generate a set of undifferentiated neurites; then a single process, the future axon, grows much faster than all the others and exceeds them in length[71]. The designated axon gradually exhibits a number of distinct biochemical and functional characteristics. Axonal marker proteins include GAP43, L1, IGF1R, TAU1 (also known as MAPT) and others[11,28,72,73]; proteins that are found selectively in the dendrites include the transferrin receptor and microtubule-associated protein 2 (REFS 28,72). In addition, axonal microtubules are oriented uniformly, with their plus ends pointing to the growth cone, whereas dendritic microtubules are oriented randomly[74].

The example of the hippocampal neuron illustrates that neurite outgrowth and plasmalemmal expansion are linked closely to the process of neuronal differentiation. But, the 'classic' neurotrophins nerve growth factor and brain-derived neurotrophic factor, which regulate differentiation[75,76], do not directly stimulate axonal membrane growth. They do, however, activate the distal transport and accumulation of the beta-subunit-containing IGF1R at the axonal growth cone, thus enabling the control of membrane expansion by IGF1 (REF. 11).

In the mature neuron, somato-dendritic membrane delivery involves the exocyst complex whereas synaptic-vesicle exocytosis in the axon's presynaptic membrane does not[77]. However, during neuronal growth the axonal growth cone depends on the exocyst for membrane expansion. Interestingly, synaptic vesicles present in growth cones take up neurotransmitter but do not seem to be competent to release it, even though the PPV fusion mechanism is functional[7]. This suggests that the final step of neuronal polarization occurs during synaptogenesis.

Membrane expansion in the dendrite. The axon's extremely elongated configuration and its rapid growth ahead of the dendrites favour the analysis of its growth mechanisms. As a result, much less information is available on dendritic growth. However, it should not be overlooked that although the growth rate of the dendritic tree cannot match that of the axon, the tree's elaboration can match the axon's dimensions.

The presence in large dendrites of stacks of rough ER and Golgi cisternae clearly distinguishes dendrites from axons. Of particular significance, the neuronal Golgi complex is organized into Golgi stacks in the perikaryon plus additional, apparently independent 'Golgi outposts' in dendritic shafts and spines[78,79]. Thus, the dendrites' capacity to synthesize specific secretory products and

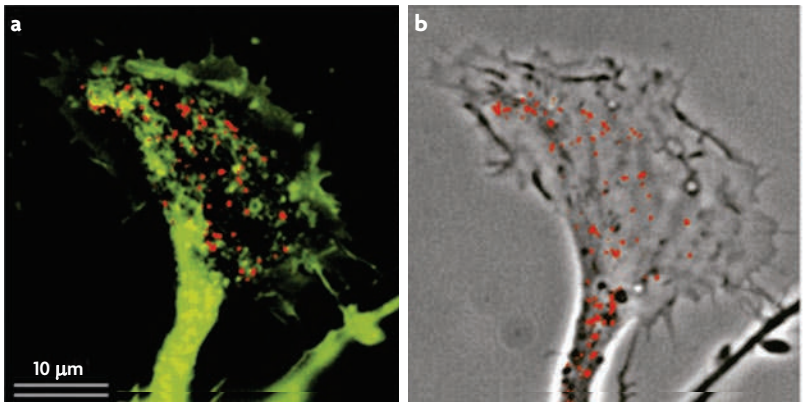


Figure 4 | **Pulse–chase labelling of a rat hippocampal growth cone in culture.** BODIPY-ceramide, a fluorescent sphingomyelin and glucosylceramide precursor (pulse: 30 min at room temperature; chase: 2.5–3 h at 37 °C), can be used to examine the arrival of Golgi-derived vesicles at the growth cone and the precursor–product relationship that these vesicles have with the plasmalemma. **a** | Golgi-derived vesicles containing a high concentration of the label emit red fluorescence; upon dilution into the plasmalemma, the label fluoresces in green. **b** | A superimposition of red-emission and phase-contrast images of the structure shown in part **a**. Figure is modified, with permission, from REF. 11 © (2003) Company of Biologists.

membrane compartments for exocytosis is far more developed than that of the axon, with dendrites emerging as semi-autonomous regions of the neuron. Accordingly, selected mRNAs, such as those that encode microtubule-associated protein 2 and calcium/calmodulin-dependent protein kinase II, are transported into the dendrite for translation into proteins[80,81].

The presence of the complete set of organelles of the secretory pathway in dendrites should enable them to generate the new membrane needed for localized plasmalemmal growth. Ultrastructural analyses of dendrites have demonstrated the presence of vesicles resembling PPVs[8,9]. More recent work has shown that some vesicular traffic from the rough ER and from the Golgi is directed into dendrites. This includes the transport of the transferrin receptor and that of a model cargo membrane protein, vesicular stomatitis virus G protein, from dendritic Golgi outposts[28,79,82]. Vesicular exocytosis is necessary for dendrite growth, as is the case for axons. The plasma membrane target SNAREs syntaxin 12 and SNAP25 are present in both axons and dendrites, and inhibition of either one or of TI-VAMP blocks both axonal and dendritic outgrowth[57,59,61,83]. However, silencing the expression of the small GTP-binding protein SAR1, which is involved in ER-to-Golgi vesicular trafficking, reduces dendritic growth preferentially. Moreover, protein kinase D is necessary for proper dendritic targeting of vesicles from the TGN and for neuronal polarization[84,85]. This suggests that although some aspects of the membrane pathways in axons and dendrites are shared, they may not be identical[86] (FIG. 3). Moreover, membrane insertion along the neurite's shaft may be more prevalent in dendrites than in axons[38]. The quantitative and qualitative contributions of Golgi outpost-derived vesicles to dendritic growth are not yet clear, but recent results indicate that Golgi outpost impairment reduces dendrite growth[82,86].

Like axons that form collateral sprouts, dendrites and in particular their spines are highly dynamic. Elaboration of the dendritic tree and increases in spine density both necessitate the expansion of the dendrite's plasmalemma. It is tempting to speculate that dendrites' elaborate secretory-pathway machinery endows them with a particular ability to carry out such structural changes. To some degree ready access to new membrane seems to depend on endosomes. In contrast to the axon, where early endosomes are confined to the growth cone, the perikaryon and dendrites of cultured hippocampal neurons contain large amounts of tubular early endosomes[87]. This suggests that endocytosis is more widespread in dendrites than in axons. Recycling endosomes store retrieved membrane from deleted spines and provide the membrane for surface expansion of growing spines, and membrane recycling to the cell surface has been shown to be necessary for activity-dependent spine enlargement[88].

The precise geometric arrangement of dendritic trees[89] implies that dendrite growth is precisely controlled. Indeed, it is regulated by numerous microenvironmental cues, including cell-adhesion molecules, neurotransmitters and other factors[90]. These factors seem to be distinct from or to act differently to those that regulate axonal growth. For example, IGF1R, which regulates axonal membrane expansion and growth, is absent from dendrites[11], and semaphorin 3A acts as a repellent for axons but as an attractant for dendrites[91].

Does membrane expansion drive the establishment of polarity? The aspects of neuronal polarity discussed so far pertain to distinctions between axon and dendrites and what these differences mean for membrane expansion. However, membrane expansion *per se* might influence neuronal polarity. Neurite length, which is highly dependent on membrane expansion, is not only the most obvious criterion for identifying the axon, it is also crucial for maintaining axonal identity, as the results of experimental axonal transection *in vitro* have shown. If the remaining axonal stump is shorter than the minor neurites, one of these will convert into an axon; if the stump is longer it will retain its axonal identity[92,93]. It has been argued that the emergence of the axon necessitates the operation of a positive feedback mechanism that maintains a high rate of outgrowth[94]. A number of mechanisms, possibly operating together[94], have been proposed. Axonal membrane expansion should also be considered as a candidate mechanism. IGF1R is essential for membrane expansion as well as for establishing neuronal polarity[11,26,46]. As increasing membrane expansion brings to the cell surface membrane components, including IGF1R, this mechanism is self-reinforcing and thus could represent the postulated positive feedback mechanism for axonal specification and neuronal polarization.

In summary, the fundamental principles of membrane biogenesis through the secretory pathway and of plasmalemmal expansion through regulated exocytosis apply to axons as well as dendrites, but important differences exist, especially with regard to the physical

separation between the membrane-synthetic machinery and the primary site of insertion (FIG. 3) and the control mechanisms (which seem to involve not only different factors but also different regulatory elements in the secretory pathway). Further differences may emerge, as we are only just beginning to understand how plasmalemmal expansion works in the dendrite.

De novo growth versus regeneration

Several conditions of neurite growth and plasmalemmal expansion can be distinguished *in vivo* (FIG. 1). In addition, membrane components must undergo turnover in established neuronal networks. *De novo* axon growth is the condition most commonly studied in cultured neurons, and such studies have provided much of the mechanistic information reviewed here. Axonal sprouts and regenerating axons bear growth cones and express a classic axonal growth and growth cone marker, GAP43 (REFS 73,95). Although differences between axonal *de novo* growth and regeneration are becoming better defined (for example, see REF. 96), in both scenarios the plasmalemma seems to expand in basically the same manner.

When the axon reaches an appropriate target, such as a dendrite, synaptogenesis proceeds. This transformative process entails cessation of growth (at least temporarily), assembly of presynaptic specializations and the gradual removal of growth cone organelles. Presynaptic components reach the developing synaptic site by axoplasmic transport from the perikaryon's TGN, and at least two types of vesicle carrier have been identified: piccolo transport vesicles (PTVs) and synaptic vesicle protein transport vesicles (STVs), both of which deliver active-zone and exocytosis-machinery proteins[97]. In a coordinated manner, neurotransmitter receptors, adhesion molecules and scaffold proteins are assembled at the dendrite's postsynaptic membrane[97,98]. Thus, specifically targeted delivery of new membrane components and their aggregation by adhesion and scaffold proteins further modify the membrane laid down by PPVs.

Once neuronal networks are established, the further elongation of processes that is required by the organism's overall growth presents a quite different situation because the focal point of membrane insertion during *de novo* growth, the growth cone, has been replaced by a presynaptic terminal. Vesicles that morphologically resemble PPVs have been seen in synapsing but still developing neuronal networks, sometimes adjacent to a synaptic contact[9,10]. They could be contributing to elongation or to emerging collateral sprouts. Once the growth cone has been transformed into a presynaptic ending there are no obvious sites of insertion of new membrane, at least none that are currently known. We can only speculate, therefore, that vesicles are transported into the neurite and incorporated into the plasmalemma by exocytotic fusion, perhaps at fusion sites that emerge at random or at multiple predetermined sites. Interestingly, the exocyst is not just concentrated in the growth cone: it can be found in spots along the growing axon[50]. The process that mediates membrane expansion in the networked neuron may also be involved in the turnover of membrane components. The only difference may be that in the case of elongation endocytosis of membrane components occurs at a low rate whereas in the case of the fully grown neurite PPV insertion and membrane retrieval must balance. Virtually nothing is known about these mechanisms *in vivo* because they are particularly difficult to study. We can predict, however, that specific components, packaged into specific vesicle types, are targeted (in some cases perhaps by transcytosis) to the various distinct regions of the neurite, for membrane expansion as well as turnover.

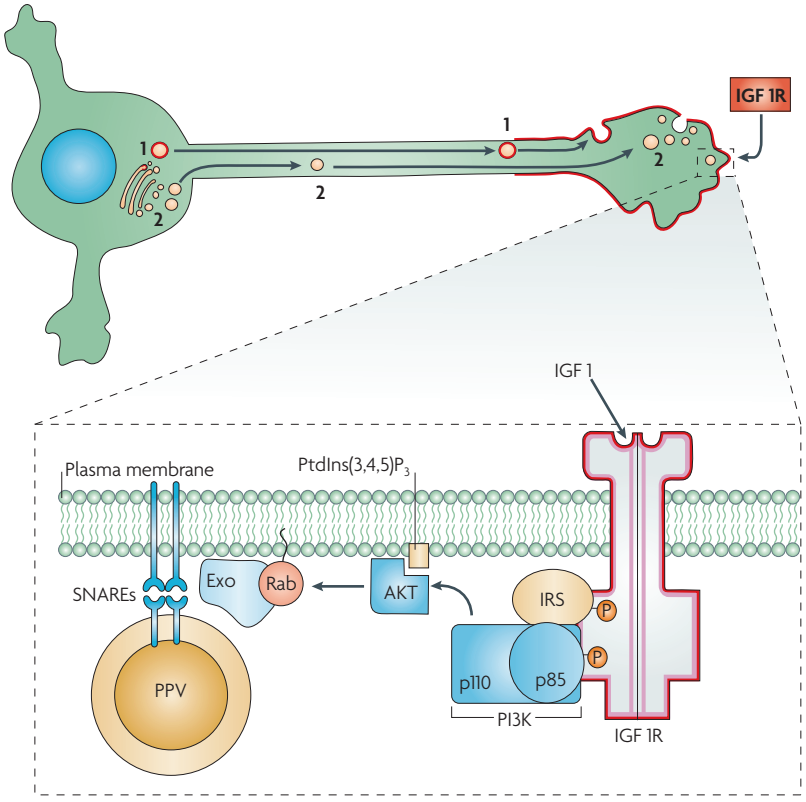


Figure 5 | **A model of the regulation of plasmalemmal expansion in the growing axon.** The growing neuron (top) synthesizes vesicles that contain insulin-like growth factor 1 (IGF1) receptor (IGF1R) and are transported to the axonal growth cone by a KIF2-dependent mechanism (process 1). Other plasmalemmal precursor vesicles (PPVs) provide the bulk of new membrane and are shipped to the growth cone by KIF2-independent transport (process 2). Stimulation of IGF1R activates phosphoinositide 3-kinase (PI3K, comprised of a p110 catalytic and a p85 regulatory subunit) and the synthesis of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3), which in turn increases AKT activity. Findings in other cell systems indicate that this might stimulate a Rab family member, which could recruit the exocyst complex (Exo) to the plasma membrane and initiate plasmalemmal precursor vesicle (PPV) docking. Activation of the fusion machinery (SNAREs (SNAP receptors)) subsequently triggers exocytosis. IRS, insulin receptor substrate.

Conclusions and perspectives

The main conclusion of the material reviewed here is that plasmalemmal expansion in the neuron uses the well-established universal cellular mechanisms of membrane biogenesis by the secretory pathway, transport to the cell periphery and exocytotic insertion (FIG. 3). Overwhelming evidence, some of which dates back many years, supports this conclusion. The adaptation

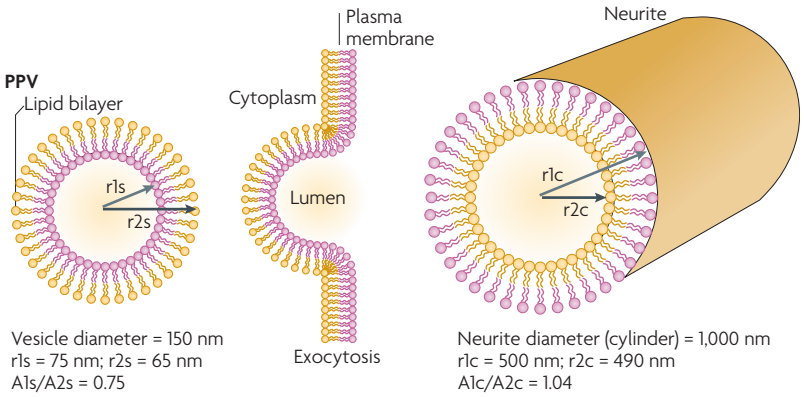


Figure 6 | **Change in membrane topology during exocytosis of plasmalemmal precursor vesicles (PPVs).** In a highly curved membrane, such as that of PPVs, the two lipid leaflets constituting the bilayer (shown in different colours) are of different size. The surface areas of the two monolayers in such spheres can be calculated based on the two radii r1s and r2s (which differ by the membrane thickness). For a sphere with a diameter of 150 nm and a membrane thickness of 10 nm, the surface (A1s) of the vesicle's luminal lipid layer is ~25% smaller than that of the cytoplasmic membrane leaflet (A2s). During exocytosis the luminal layer becomes the external leaflet of the neurite's plasma membrane, which essentially forms a cylinder of ~1000 nm diameter. The radius of this cylinder (r1c) is larger than that of the cylinder formed by the cytoplasmic leaflet, and so the surface areas A1c and A2c differ by ~4%. Owing to the change in configuration, the PPV's luminal leaflet would have to increase in area by ~30% if the cytoplasmic leaflet remained the same.

of these fundamental processes to the extreme needs of a growing neuron is remarkable. The necessary special modifications include the targeting of specific vesicle types to axons versus dendrites, the long vesicle transport routes in the axon and the growth factor-regulated mechanism of insertion at the growing tip. In addition, dendrites containing Golgi outposts have emerged as semi-autonomous structures that are capable, to some degree, of synthesizing their own membranes and membrane proteins as well as apparently controlling vesicle traffic with a set of regulatory proteins that differs partially from that which operates in axons. Although these phenomena are well established for neurites growing *de novo*, little is known about plasmalemmal expansion (and turnover of membrane components) in networked axons and dendrites. We can only speculate that this might involve a vesicle export and exocytosis mechanism, and perhaps insertion throughout the growing neurite. The dimensions of membrane expansion and the complexity of the mechanisms involved suggest that the huge increase in surface (and surface/volume ratio) could be a rate-limiting step of neuronal growth.

Membrane growth and disease. Alterations in the secretory pathway are likely to affect plasmalemmal biogenesis and expansion. Such defects interfere with many cellular functions and so afflicted embryos are unlikely to survive[99]. However, because of the extreme and specialized demands of neuronal membrane growth, relatively small losses of function in the pathway from synthesis to insertion might interfere with neuronal membrane expansion and nervous system function selectively, without critically affecting other organs. Cellular longevity also might allow small deficits to have a greater cumulative effect on neurons than other cell types. It is possible, therefore, that some nervous system disorders of unknown aetiology are the result of defects in plasmalemmal expansion and/or turnover.

One candidate disorder is Huntington's disease, a neurodegenerative disorder caused by unstable expansion of CAG repeats in the coding region of huntingtin[100]. The resulting enlarged stretch of glutamine residues near the amino terminus of mutant huntingtin seems to interfere with its normal function(s) as well as cause a toxic gain of function[101]. Interestingly, huntingtin is associated with endocytic and secretory vesicles and indirectly binds to the small GTPase RAB8, which is involved in vesicular trafficking[102,103]. Huntingtin also associates with different microtubule motor complexes[104–107]. A defect in huntingtin therefore might interfere with the growth and maintenance of the neuronal plasma membrane and eventually cause cell death[108,109]. However, huntingtin is a large protein (~350 kDa) with over 200 potential binding partners and so its mutation is likely to affect various cellular mechanisms. Deficits in several of these may contribute to the pathology of the disease[100].

Another candidate disorder of membrane expansion and turnover is Charcot–Marie–Tooth disease (CMT), a family of peripheral neuropathies that includes inherited axonopathies. Some axonopathies have been linked to mutations in proteins involved in membrane trafficking, such as the microtubule motor KIF1B, dynactin and the small GTPase RAB7 (REF. 110). Hereditary spastic paraplegias (HSPs) have been linked to mutations in a gene of the dynamin superfamily, *atlastin 1*, which encodes a small GTPase that is involved in membrane trafficking and is necessary for axon formation[111,112]. Thus, the pathogeneses of CMT and HSPs are also likely to involve impaired membrane trafficking. It does not seem to be a coincidence that these two disorders affect primarily neurons with long axons[111,113]. The very large surface of these cells requires effective membrane expansion and maintenance. It should be kept in mind, however, that membrane expansion and turnover are not the only processes that are dependent on membrane trafficking mechanisms.

What next? Despite the substantial advances in our understanding of plasmalemmal expansion, a number of questions remain unanswered. First, it is evident that the neuron produces several vesicle types for axonal and dendritic targeting, for secretion (including synaptic vesicles) and for membrane expansion. How many different vesicle types are being produced? During later stages of axonal outgrowth at least three different types of PPV are formed (transported by KIF2, KIF4 and kinesin, respectively; see above), plus dendritic PPVs, PTVs, STVs, large dense-core vesicles and synaptic vesicles. How are their synthesis, targeting and exocytosis kept apart and regulated individually? Second, it seems reasonable to postulate that the demand for PPVs at the growth cone must be coordinated with the supply from the perikaryon. The wide separation of sites of membrane synthesis and

insertion in axons would seem to necessitate specialized signalling mechanisms. How is increasing or decreasing demand for membrane synthesis and transport communicated from the growth cone to the perikaryon? Third, what is the mechanism of plasmalemmal expansion in the networked neuron? Are there specific sites of membrane insertion? As the growth cone is no longer crawling forward, what stimulates expansion? Could stretching of the axon due to growth of the organism trigger PPV exocytosis[114]? Fourth, to what degree is the process of plasmalemmal expansion involved in axonal specification? Is it just a read-out of polarity or is it an essential step in the establishment of neuronal polarity? An affirmative answer to this last question would add a quite unexpected dimension to the phenomenon of membrane biogenesis in the neuron.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
κ-opioid receptor | ARF6 | *atlastin 1* | EPHA2 | EXO70 | EXO84 | GAP43 | huntingtin | IGF1 | IGF1R | KIF1B | KIF2 | KIF4 | L1 | NCAM | RAB8 | RAB11 | RALA | RALB | SAR1 | SEC3 | SEC5 | SEC6 | SEC8 | SEC10 | SEC15 | semaphorin 3A | SNAP25 | syntaxin 3 | syntaxin 12 | TAU1 | TC10 | TI-VAMP | VAMP2

OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
Huntington's disease

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