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Pathways for axonal targeting of membrane proteins

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Abstract

The distribution of proteins to the correct domains of neurons is crucial for neuronal functioning. Here I discuss possible mechanisms underlying sorting to the axonal plasma membrane which differ with respect to the steps providing selectivity for axonal vs. somatodendritic cargo. Selectivity can be executed at one or more than one step, including sorting into distinct carriers in the Golgi, preferential transport along axonal microtubules, fusion with the plasma membrane, and importantly retrieval by endocytosis. A protein might in fact be sorted multiple times at different places to achieve axonal localization.

Keywords: Axonal targeting; Selectivity; Endocytosis; Retention; Trans-Golgi network

1. Introduction

Vertebrate neurons are highly polarized cells possessing different processes, namely axons and dendrites. These two kinds of processes fulfill different functions: dendrites are primarily responsible for signal reception and axons for signal generation and propagation. In order to fulfill these distinct functions, axons and dendrites are morphologically and molecularly distinct. Exactly how somatodendritic and axonal membrane proteins accumulate in the correct domains is less than clear, but some sort of targeting mechanisms must exist (Foletti et al., 1999; Horton and Ehlers, 2003; Winckler and Mellman, 1999).

2. Selectivity filters for polarized transport to the axon

Membrane proteins, which mature through the ER and Golgi, reach the plasma membrane from the trans-Golgi network (TGN). The transport from the TGN to the plasma membrane consists of multiple steps, ❶ packaging of cargo proteins into post-Golgi carriers, ❷ transport along microtubules, and ❸ fusion with the plasma membrane. Each of these steps could serve as a selectivity filter to sort axonal from somatodendritic cargo and could be a target for differential regulation (Fig. 1A). A first selectivity filter (❶) could exist at the TGN and provide preferential inclusion of axonal cargo molecules into an axonally-targeted post-Golgi carrier (blue circles) which would largely exclude somatodendritic cargo (red circles) and vice versa. In a second selectivity filter, axonal carriers could preferentially associate and travel on axonal microtubules (❷) and thereby be excluded from dendrites. A third selectivity filter could restrict fusion of axonal carriers to the axonal plasma membrane (❸), so that axonal carriers which aberrantly traveled into dendrites would be unable to fuse with the somatodendritic plasma membrane. In addition, post-insertion mechanisms could contribute to the polarized distribution of membrane proteins (Fig. 1B). For instance, missorted proteins could be selectively removed from the incorrect domain by endocytosis (selectivity filter ❹) and resorted or degraded, while proteins in the correct domain could be selectively tethered there. A priori, not all of these steps have to be differentially executed for axonal and somatodendritic proteins, and it suffices for successful axonal accumulation if a subset of these steps is selective in each case. In fact, it is likely that different axonal proteins use different axonal pathways in which selectivity filters are operative at different steps. Evidence is accumulating from several labs that not all axonal proteins use the same

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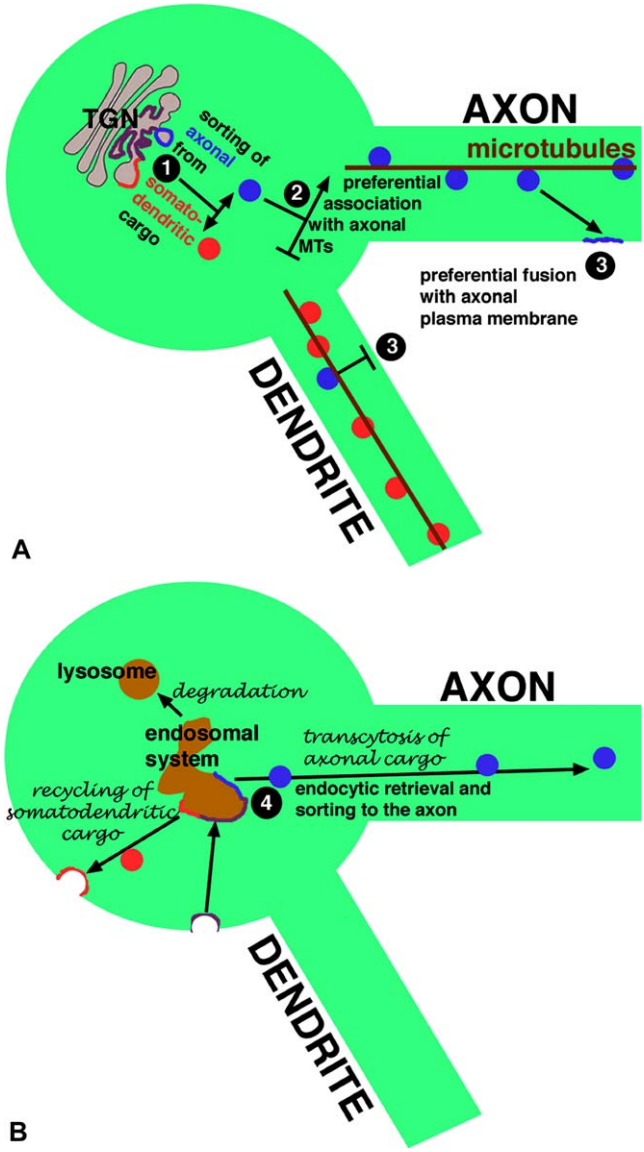

Fig. 1. (A) Pre-insertion selectivity filters. (B) Post-insertion selectivity filters.

mechanisms to achieve axonal accumulation (Burack et al., 2000; Garrido et al., 2001; Nakata and Hirokawa, 2003; Sampo et al., 2003; Wisco et al., 2003).

3. Models for axonal targeting

Based on the arguments above, one can envision several models for axonal targeting. These models differ from each other with respect to the transport and/or post-insertion steps that provide selectivity. The first would be a direct axonal pathway. Axonal cargo would be sorted into axonally-targeted post-Golgi carriers (selectivity filter ❶ ON) which would travel directly to the axon (selectivity filter ❷ ON) and fuse with the axonal plasma membrane. In this model, the selectivity filters would occur at the level of TGN packaging as well as at the level of preferential transport into axons. Subsequent steps (fusion, endocytosis, retention) might or might not be selective.

What would happen if selectivity filter ❶ is ON, but selectivity filter ❷ is OFF? In this model, selective packaging into axonally-directed post-Golgi carriers would occur at the level of the TGN (selectivity filter ❶ ON), followed by non-selective transport into both axons and dendrites (selectivity filter ❷ OFF). If selectivity filter ❸ is ON, axonal accumulation can still be achieved by controlling domain-specific fusion with the plasma membrane. Axonal carriers would be incompetent to fuse with the somatodendritic plasma membrane and only be capable of fusing with the axonal plasma membrane (selectivity filter ❸ ON). This would be a selective fusion model (Burack et al., 2000).

What would happen if selectivity filter ❶ is OFF? Since cargo packaging is non-selective (selectivity filter ❶ OFF), post-Golgi carriers would contain both axonal and somatodendritic cargo. Therefore, all pre-insertion steps, i.e. packaging, transport, and fusion, would be non-selective (selectivity filters (1–3) OFF). Axonal accumulation could still be achieved if post-insertion selectivity filters are ON, i.e. selective removal of axonal cargo from the incorrect somatodendritic domain by endocytosis (selectivity filter ❹ ON). This model is therefore based on domain-specific endocytosis (Garrido et al., 2001).

These different models for axonal targeting cannot be distinguished by looking only at the steady-state distribution of proteins and many labs have turned to live imaging of GFP fusion proteins, to kinetic analysis, and to endocytosis assays (Burack et al., 2000; Garrido et al., 2001; Sampo et al., 2003; Wisco et al., 2003; Kaether et al., 2000; Nakata and Hirokawa, 2003; Nakata et al., 1998). Since not all axonal proteins necessarily reach the axon by the same pathway, detailed analysis of individual axonal proteins needs to be undertaken and evaluated. What evidence have these studies provided for the existence of the different selectivity filters?

4. Experimental evidence for selectivity filter ❶ : sorting at the TGN

Generation of axonal cargo-enriched transport carriers (selectivity filter ❶) is presumed to occur in the TGN and to require signals in the axonal cargo proteins which can mediate preferential inclusion into axonal carriers. In non-neuronal cells, sorting of two different cargo molecules into distinct post-Golgi carriers has been shown both biochemically (Wandinger-Ness et al., 1990) as well as by dual fluorescent live imaging (Keller et al., 2001). In neurons, live imaging has demonstrated that the somatodendritic transferrin receptor travels preferentially into dendrites and is excluded from entering axons (Burack et al., 2000). Presumably, axonal cargo is not contained in these same carriers. Hirokawa and colleagues recently showed that several axonal cargo molecules travel in the same carrier into axons (Nakata and Hirokawa, 2003). Again, presumably somato-

dendritic cargo is not contained in these same carriers. We clearly need more simultaneous imaging of axonal and somatodendritic cargoes leaving the TGN to strengthen the likely case for TGN sorting of axonal and somatodendritic cargoes away from each other. Interestingly, there is also evidence that some axonal cargoes travel anterogradely in the axon in distinct carriers (Kaether et al., 2000), raising the question of how many distinct axonally-directed post-Golgi carriers are generated in a neuron. Additionally, there is evidence from non-neuronal cells that the TGN might not be the last sorting compartment before the plasma membrane is reached. Several groups have described the generation of long tubular carriers which emerge from the TGN. Cargo contained within these rather large tubules appears to be sorted further from within these carriers (Jacob and Naim, 2001; Polishchuk et al., 2003; Polishchuk et al., 2000; Puertollano et al., 2003). Also, some biosynthetic cargoes appear to travel from the TGN via recycling endosomes to the plasma membrane (Folsch et al., 2003). Additional sorting therefore could take place in the recycling endosome as well as in post-Golgi tubular carriers.

5. Experimental evidence for selectivity filter ❷: preferential transport into the axon

Once post-Golgi carriers have been generated, they need to translocate to the plasma membrane to undergo exocytosis. Given the long distances, this translocation is active and takes place along microtubules via microtubule-based motors, mostly from the kinesin family (KIF). Different KIF members are thought to specifically transport subsets of organelles to their final destinations (Gunawardena and Goldstein, 2004; Terada and Hirokawa, 2000). It is therefore posited that KIFs have the capability to bind specifically to the correct post-Golgi carriers. Several labs have obtained evidence for such a notion and have demonstrated specific binding of certain membrane proteins to certain KIFs (Kamal et al., 2000; Nakagawa et al., 2000).

A second question is how the KIFs "know" where the axon is, or in fact if they "know" (Shah and Goldstein, 2000). Since microtubules are oriented with uniform polarity in axons (plus ends distal towards the terminal), axonal carriers need to use plus-end directed motors for transport. Dendrites, on the other hand, possess microtubules with mixed polarity (Baas, 1998) and, a priori, either plus-end or minus-end directed motors could transport cargo carriers into dendrites. From first principles, specific transport of somatodendritic cargo into dendrites could be achieved if dendritic anterograde transport uses minus-end directed motors. Erroneous transport into axons would be impossible because of the plus-end-distal orientation of axonal microtubules. In fact, the somatodendritic transferrin receptor traveled exclusively into dendrites and was found not to enter axons (Burack et al., 2000). Whether the KIF which transports transferrin receptor-containing carriers into dendrites is in fact a minus-end directed motor is currently unknown.

Plus-end directed motors, on the other hand, could in principle travel into both dendrites and axons. This raises the question of whether there is in fact preferential transport of axonal carriers into axons or does selectivity filter ❷ not exist? Selectivity filter ❷ requires a "smart" motor (Setou et al., 2002; Shah and Goldstein, 2000), i.e. a motor which "knows" something in addition to where the plus end of a microtubule is: A "smart" motor needs to distinguish axonal from dendritic plus-end-distal microtubules. Carriers containing the axonal cell adhesion molecule L1/NgCAM, for instance, translocate bidirectionally in both dendrites and axons (Burack et al., 2000), suggestive of non-specific transport into both axons and dendrites, using a "dumb" motor. On the other hand, the plus-end directed KIF21B is restricted to dendrites while KIF21A is also axonal (Marszalek et al., 1999). Hirokawa and colleagues recently showed that multiple axonal cargoes are carried in the same carrier (Nakata and Hirokawa, 2003). These axonal carriers show preferential transport into the axon, implicating a "smart" motor. These authors showed that KIF5 was the motor responsible and that KIF5 had the capacity to preferentially recognize the microtubule population in the axonal initial segment. The molecular basis for this recognition is currently unknown. Hirokawa's group also provided evidence that accessory proteins associating with KIFs might provide the molecular mechanisms to make the motor smart (Setou et al., 2002). At least for some axonal carriers, therefore, selectivity filter ❷ appears ON, enabling preferential transport along axonal microtubules. It is important to note, though, that while the majority of axonal carriers were observed to directly enter the axon from the TGN region in this study (Nakata and Hirokawa, 2003), a substantial number nevertheless erroneously entered dendrites instead.

6. Experimental evidence for selectivity filter ❸: domain-specific fusion

Based on the observation that carriers containing the axonal NgCAM molecule are transporting inside dendrites, Banker and colleagues have proposed that directed transport into axons does not occur, but rather that the fusion capability of NgCAM-carriers is domain-specific (Burack et al., 2000; Silverman et al., 2001). Since, it is technically challenging to directly demonstrate in a quantitative fashion where fusion of a particular biosynthetic cargo occurs in a neuron, evidence supporting domain-specific fusion so far is largely indirect (Sampo et al., 2003). Since fusion with membranes involves SNARE complexes, axonal- or dendritic-specific SNAREs could provide the molecular basis for such a selective fusion function (Foletti et al., 1999). Many of the SNAREs occur as large gene families which show highly specific subcellular localizations. Currently, it is unknown which SNAREs, if any, might contribute to the specificity for axonal- or dendritic-specific fusion. Additionally, other proteins regulate the basic SNARE machinery and might be crucial for

controlling fusion specificity (Gerst, 2003). Interestingly, Galli and co-workers recently showed (Martinez-Arca et al., 2004) that synaptobrevin (VAMP2) mediates fusion with both dendritic and axonal plasma membrane. Therefore, direct experimental evidence for a domain-specific fusion machinery is still elusive.

7. Does endocytosis contribute to axonal targeting (selectivity filter ❹)?

I presented above some of the evidence currently available for the existence of selectivity filters at the level of TGN sorting, preferential transport, and preferential fusion. Each of these filters is likely not 100% efficient leading to some missorting of axonal cargo to the somatodendritic domain. It is possible, even likely, that some of this missorted material found in the incorrect domain is removed from the plasma membrane by endocytosis. Endocytosis could therefore play a kind of salvage role, a secondary back-up mechanism to tidy up the occasional missorted proteins which escaped from selectivity filter (1–3). It might in fact be prudent to think about axonal targeting as a multi-step pathway where sorting is iterative and occurs through the sequential execution of multiple less-than-perfect sorting steps.

In contrast to this "endocytosis-as-salvage" idea, Benedicte Dargent's lab presented evidence for domain-specific endocytosis as a primary mechanism of generating axonal accumulation (Garrido et al., 2001). These authors found that the "axonal targeting sequence" of an axonally-accumulated chimeric receptor mapped to an endocytosis motif. Mutations of two crucial residues in this endocytosis motif led to inhibition of endocytosis and loss of axonal polarization. Interestingly, Gary Banker's group reported subsequently that the axonal localization of VAMP2 similarly was compromised by mutation of its endocytosis motif (Sampo et al., 2003). In my own lab, we found that downregulation of endocytosis using dominant-negative dynamin led to loss of axonal polarization of L1/NgCAM (Wisco et al., 2003). These studies together suggest that endocytosis might contribute more widely to axonal targeting.

How might domain-specific endocytosis be achieved? Currently, no evidence exists to my knowledge for domain-specific endocytosis signals and/or domain-specific endocytosis adaptors. NaK-ATPase in MDCK cells, for instance, is specifically retained on the basolateral side by direct interaction with domain-specific ankyrin-based membrane skeleton (Nelson and Veshnock, 1987). Molecules on the incorrect domain, on the other hand can be endocytosed. This suggests a model in which domain-specific binding to cytoskeletal elements could inhibit recruitment to coated pits and subsequent endocytosis. Another mechanism might be in effect for L1. Vance Lemmon's group showed that phosphorylation of the endocytosis signal inhibits binding of the clathrin adaptor AP2 (Schaefer et al., 2002). Domain-specific phosphorylation events could therefore regulate where endocytosis of L1 takes place.

8. Role of endocytosis in axon outgrowth?

During axon outgrowth, large amounts of new membrane and associated proteins are inserted in the axon to support expansion (Futerman and Banker, 1996). The molecular machinery supporting this membrane addition appears to be, at least in part, distinct from the machinery supporting synaptic vesicle fusion. The group of Thierry Galli, for example, described a toxin-insensitive VAMP (TI-VAMP, a.k.a. VAMP7) which is required for axon outgrowth but not synaptic vesicle fusion (Martinez-Arca et al., 2000). Surprisingly, TI-VAMP localizes to an endocytic compartment (Alberts and Galli, 2003; Alberts et al., 2003). Similarly, inhibition of the endocytic syntaxin13 impairs axon outgrowth (Hirling et al., 2000). What essential role might endocytosis play in axon outgrowth? One possibility is that receptor recycling is necessary for outgrowth because it underlies the function of adhesion receptors. L1, for instance, appears to be inserted at the margin of the growth cone, endocytosed at the palm of the growth cone, and recycled back to the margin (Kamiguchi, 2003; Kamiguchi and Lemmon, 2000). Endocytosis of the adhesion machinery might be crucial for orchestrating local cycles of adhesion and detachment which enable protrusion. In this scenario, molecules like TI-VAMP and syntaxin13 might act locally in axonal growth cone-associated endosomes (Alberts and Galli, 2003).

A second possibility is that new material is transported from the endosomal system for bulk addition of membrane at the axonal growth cone. Why would new material be even found in the endosomal system? It could be that receptors "salvaged" by endocytosis from the incorrect domain (as described above) are transported from the somatodendritic domain to the axon and participate in growth, rather than being degraded. In this scenario, molecules like TI-VAMP and syntaxin13 might act at distant somatodendritic endocytic sites and might regulate a route from the somatodendritic domain via the endocytic system to the axon.

9. Transcytosis in neurons—transport from dendrites to axons (or vice versa) via endocytosis

In epithelia, some membrane proteins are transported from one plasma membrane domain to the other via the endocytic system, a process termed transcytosis. Do neurons possess similar transport pathways which connect the dendritic and axonal surfaces via endocytosis? Several receptors are endocytosed at distal axonal sites and transported retrogradely to the soma where they participate in signaling events (Ginty and Segal, 2002; Barker et al., 2002). Also, the infection route of some viruses is transcytotic: infection and uptake take place at axonal terminals and the endocytosed viral particles are transported retrogradely to the soma (Kuypers and Ugolini, 1990). The opposite pathway (dendrite-to axon transcytosis) is less well described, but

some evidence for its existence has been presented. For instance, the polymeric Ig receptor (pIgR) is inserted somatodendritically first and then transcytoses to the axon (De Hoop et al., 1995), similar to its transcytotic trafficking in epithelial cells (Luton and Mostov, 1999). Neurotrophins also can be taken up by the dendrites of retinal ganglion cells in the retina and travel to the axon by transcytosis (Von Bartheld, 2004). Dendroaxonal transcytosis of transferrin has also been reported (Hemar et al., 1997).

Interestingly, in some epithelial cells, such as hepatocytes, transcytosis is the primary sorting pathway for most apical-resident membrane proteins (Tuma and Hubbard 2003). Recent surprising evidence shows that at least some GPI-linked proteins reach the apical domain by transcytyosis even in MDCK cells (Polishchuk et al., 2004), which are routinely considered to be primarily TGN sorters. Similarly, my lab has presented evidence that in neurons transcytosis might be able to provide an axonal delivery pathway for certain axonal membrane proteins (Wisco et al., 2003) and not function just as a salvage pathway for missorted proteins. Our studies on the kinetics of NgCAM delivery suggest a model in which the initial insertion of NgCAM occurs in the somatodendritic domain, followed by endocytosis and transport to the axon (Wisco et al., 2003).

What is the evidence that NgCAM transcytosis is not just a salvage pathway to resort missorted NgCAM molecules? What is the evidence that NgCAM axonal accumulation does not occur by uniform delivery coupled to endocytosis, as proposed for VGNC chimeras (Garrido et al., 2001) and VAMP2 (Sampo et al., 2003)? We found that mutations in the cytoplasmic tail of NgCAM destroy transcytotic axonal transport, leading instead to direct routing to the axon. Therefore, both direct and indirect pathways exist in polarized neurons. Since a single point mutation in the NgCAM cytoplasmic tail leads to direct axonal targeting, we propose that the initial somatodendritic delivery is domain-specific and signal-mediated (Wisco et al., 2003). Uniform insertion into both axonal and somatodendritic domains would likely not be disrupted by a single point mutation. Transcytosis is therefore a 4th possible pathway for axonal transport (Fig. 1B).

Banker and colleagues have proposed a different model for NgCAM targeting to the axon (Sampo et al., 2003) which is based on domain-specific fusion. How can these two sets of data be reconciled? It is possible that multiple pathways can be used by the same protein depending on cell type, density, stage of maturation, or other currently unrecognized factors. More work is required to fully elucidate the axonal targeting pathway of NgCAM.

10. Conclusion

Based on evidence from multiple groups, we propose that axonal targeting might be a multi-step pathway where sorting is iterative and occurs through the sequential execution of multiple less-than-perfect sorting steps. Importantly, different axonal proteins might rely to different degrees on the selectivity of different transport steps. This kind of pathway organization could serve to provide cells with considerable plasticity in regulating pathway choices. When and where cells might in fact modulate pathway choices and what role this might play physiologically remains to be discovered.

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