Trafficking Guidance Receptors

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Wiring of the brain relies initially on the correct outgrowth of axons to reach the appropriate target area for innervation. A large number of guidance receptors present in the plasma membrane of axonal growth cones and elsewhere on the neuron read and execute directional cues present in the extracellular environment of the navigating growth cone. The exact timing, levels, and localization of expression of the guidance receptors in the plasma membrane therefore determine the outcome of guidance decisions. Many guidance receptors are localized in exquisitely precise spatial and temporal patterns. The cellular mechanisms ensuring these localization patterns include spatially accurate sorting after synthesis in the secretory pathway, retrieval of inappropriately expressed receptors by endocytosis followed by degradation or recycling, and restriction of diffusion. This article will discuss the machinery and regulation underlying the restricted distribution of membrane receptors, focusing on the currently best-studied example, the L1 cell adhesion molecule. In addition to the long-range mechanisms ensuring appropriate localization, the same mechanisms can act locally to adjust levels and localization of receptors. These local mechanisms are regulated by ligand binding and subsequent activation of local signaling cascades. It is likely that the localization of all guidance receptors is regulated by a combination of sorting, retrieval, recycling and retention, similar to the ones we discuss here for L1.

AN ESSENTIAL ROLE OF MEMBRANE TRAFFIC IN THE GUIDANCE AND FUNCTION OF NEURONS

The long-range conduction of electrical signals in the nervous system is exclusively performed by neurons. To accomplish this task, neurons have evolved a number of specialized features, such as a highly asymmetric cell shape with long, polarized processes extending from a cell body that is otherwise similar to that of nonneuronal cells. During development, newly born neurons migrate to their correct positions and extend axons toward their target cells (Kriegstein, 2005). These targets can be many millimeters or even centimeters away, and multiple levels of guidance mechanisms ensure the correct outgrowth of axons. Molecular cues in the environment provide guidance, and receptors recognizing these cues are present on axons to read and execute these cues (O'Donnell et al. 2009; Tessier-Lavigne 2002). In addition, guidance receptors become important again in recovery from injury during axonal regeneration (Koeberle and Bahr 2004). Less well

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understood, but no less important, are the roles some guidance receptors continue to play in synapse formation and synapse function (Godenschwege et al. 2006; Sahay et al. 2005; Tai et al. 2008). To carry out their essential activities, however, guidance receptors must be localized correctly to axons or dendrites (Allen and Chilton, 2009), and the mechanisms controlling the polarized traffic of these receptors are crucial to the proper wiring of neuronal circuits. In other polarized cell types, defects in polarized receptor traffic cause a variety of pathologies; one can expect the same in the nervous system (Olkkonen and Ikonen 2006).

LONG-DISTANCE MECHANISMS FOR AXONAL ENRICHMENT OF RECEPTORS

The correct targeting of membrane proteins is a formidable problem in all cell types, but this problem is made more challenging in neurons because they are typically many-fold larger than most other cells. For instance, a typical epithelial cell is 20–30 μm tall. The cell body of a typical neuron is on the same order in size, but the dendrites are frequently hundreds of micrometers in length, and the axon can be many centimeters up to one meter (Horton and Ehlers 2004). The huge size of the neuron coupled with the remarkably asymmetric distribution of membrane and cytosolic components (Craig and Banker 1994) multiplies the challenges for correct trafficking of membrane proteins.

The mechanisms controlling the differential distribution of membrane proteins to distinct membrane domains has been widely studied in polarized epithelial cells (Mellman and Nelson 2008). In neurons similarly, several mechanisms contribute to the nonhomogeneous distribution of receptors in the axonal versus somatodendritic plasma membrane (Lasiecka et al. 2009):

1. Direct polarized delivery: Proteins can be directly targeted to axons or dendrites from the secretory pathway. In the case of somatodendritic targeting, this mechanism typically involves the recognition of targeting signals encoded in a membrane protein's cytoplasmic domain. It also requires that a diffusion barrier exist between the axonal and somatodendritic domains.
2. Indirect polarized delivery by transcytosis: Membrane proteins may be inserted in one domain, but then redistributed following internalization and polarized transcytosis to the appropriate membrane domain. This mechanism has been established for the axonal cell adhesion molecule L1/NgCAM, which first appears at the somatodendritic surface before axonal delivery.
3. Nonpolarized delivery and selective retention: Membrane proteins may be randomly inserted and achieve asymmetric distributions by diffusing laterally until they bind to appropriately placed cytoskeletal scaffolds, or binding sites on adjacent cells, that retain them at the desired location. This retention mechanism can be coupled to preferential endocytosis of misplaced pools of the receptor and subsequent degradation.

These mechanisms can act alone or in combination to ensure the correct steady-state distribution of any given protein. For most guidance receptors, relatively little is currently known about their trafficking. We will discuss the axonal cell adhesion molecule L1 (NgCAM in chick) as the primary example to illustrate the underlying principles, because trafficking of L1 is arguably the best understood among all adhesion molecules and guidance receptors. L1 plays a role in promoting axon outgrowth, but also influences pathfinding outcomes. Mutations in L1 causes MASA syndrome in humans, characterized by mental retardation, hydrocephalus, and hypoplasia of several major axon tracts (Maness and Schachner 2007). L1 knockout mice show similar defects (Kamiguchi et al. 1998a). Although most of our focus in on L1, at the end we briefly discuss other guidance receptors, particularly the Robo receptors.

Elaboration of a Neuronal-Specific Polarized Membrane Trafficking System

The basic features of polarized membrane transport were defined by study of polarized

epithelial cells. Recent work in invertebrate and vertebrate systems has uncovered neuronal adaptations to the secretory pathway that might serve to handle the special challenges of neurons. The endosomal system in neurons similarly has adapted to neuronal-specific demands.

Neuronal Adaptations to Secretion

Neurons elaborate not just a central Golgi complex near the microtubule-organizing center in the soma, but also contain dispersed Golgi elements throughout dendrites (reviewed in Hanus and Ehlers 2008). These dendritic Golgi compartments have been named "Golgi outposts" or "satellites" (Horton and Ehlers 2003; Pierce et al. 2000). Golgi outposts are also endowed with a TGN component (Pierce et al. 2001; Tang 2008). Mobile carriers containing newly synthesized membrane proteins move bi-directionally along dendrites on ER exit, and fuse both into the somatic Golgi as well as into Golgi outposts (Horton and Ehlers 2003). The outposts are concentrated selectively at dendritic branchpoints and oriented toward the longest dendrite of pyramidal neurons, but excluded from axons (Horton et al. 2005). *Drosophila* neurons also contain dendritic Golgi outposts (Ye et al. 2007), much like those described in mammalian neurons (Horton et al. 2005). The data suggest that the outposts are dynamic and are important for dendritic arborization (Ye et al. 2007).

Dispersed Golgi elements might synthesize certain membrane cargoes locally near the sites where they will be needed. This local synthesis might thus allow for the coordination with local signaling events, either during dendrite outgrowth or later in response to synaptic activity. It is currently unknown whether guidance cues or receptors are secreted from Golgi outposts. The possibility that axons also contain some secretory compartments and might synthesize membrane proteins locally is currently only supported by scarce data, but future work will bring more light to this tantalizing prospect (Hengst and Jaffrey 2007; Lee and Hollenbeck 2003; Merianda et al. 2009; Willis et al. 2005; Yao et al. 2006).

Recent data suggest that the transport pathways and machinery for axonal and dendritic cargos are distinct. For instance, mutations affecting proteins encoded by three genes involved in ER-to-Golgi transport (Sar1, Sec 23, and Rab1) specifically disrupted outgrowth of dendrites (Ye et al. 2007), but axon outgrowth was normal. Similarly, inhibition of protein kinase D affected only dendrite but not axon outgrowth (Horton et al. 2005). How it is possible that axonal cargos can escape a block in ER-Golgi traffic is unknown. So the plot thickens as to how neurons differentially traffic cargos to axons and dendrites and the possibility of local compartments and mechanisms remains an intriguing possibility.

Neuronal Adaptations to the Endosomal System

Endosomes in neurons are not as well characterized as they are in nonneuronal cells, and are typically defined operationally as the endosomal structure to which a particular neuronal receptor is internalized (reviewed in Lasiecka et al. 2009; Schmidt and Haucke 2007). In this vein, the best studied endosomes in neurons are those in the synaptic terminal involved in synaptic vesicle recycling, those carrying out retrograde transport of neurotrophic signals, and those at dendritic spines involved in recycling AMPARs (reviewed in Howe and Mobley 2004; Kennedy and Ehlers 2006; Schweizer and Ryan 2006). For all other sites and other cargo molecules, little is known. It is clear that the endosomal system in neurons is much more diverse than that of fibroblasts and contains unique compartments in particular locations of the cell. From work in MDCK cells, we know that recycling endosomes of polarized and nonpolarized cells differ in their sorting ability, and in their recruitment of rab proteins and adaptors (Thompson et al. 2007). Neuronal endosomes, therefore, likely need to be "polarized" as well to accomplish diverse sorting and recycling tasks. Much work is still needed to delineate how neuronal endosomes are organized and regulated. It is clear, though,

that striking differences exist between axonal and somatodendritic endosomes (Mundigl et al. 1993). For instance, the early endosomal regulator EEA1, a rab5 effector thought to be essential for fusion of early endosomes, is only present on somatodendritic endosomes and not in axonal endosomes (Wilson et al. 2000). This may suggest some significant differences between the tethering or fusion machineries used by endosomes within these two neuronal domains.

In addition, a large number of distant locations in the neuron require endosomes and it is currently not known to what degree these different endosome populations are distinct and to what extent cargos in these different places can intermix. In nonneuronal cells, recycling endosomes are frequently found in a tight cluster near the centrosome. In neurons, some clustering of recycling endosomes perinuclearly can also be observed, but large numbers of endosomal compartments with characteristics of recycling endosomes are also found dispersed far into distal regions of the dendritic arbor (for instance Thompson et al. 2007). Recycling endosomes in axons are poorly characterized.

Interestingly, many membrane trafficking regulators are highly enriched in the brain or even expressed in a brain-specific fashion. For instance, the neuronal early endosome protein NEEP21 is expressed primarily in neurons and found in a distinct early endosomal population, which accumulates L1/NgCAM and AMPA receptors, but not transferrin (Steiner et al. 2005; Steiner et al. 2002; Yap et al. 2008b). Because much of the endosomal system in nonneuronal cells was initially defined by characterizing the trafficking of a small number of cargos (such as transferrin), endosomes that do not flux transferrin are currently not well understood. It is therefore likely that neurons contain a more elaborate endosomal system that makes use of common regulators and mechanisms and adapts them to specific neuronal functions by adding neuron-specific components. Delineating the components and their neuronal roles is one of the challenges in the field.

Trafficking Pathways to the Axon

Multiple routes exist for accumulation of axonal membrane proteins on the axonal surface:

Biosynthetic Sorting in the TGN and Direct Transport to the Axon

Axonal cargos, which are directly transported to axons, need to be first segregated and packed into different carriers, presumably at the level of the TGN, and then separated from those carriers destined for the somatodendritic domain and targeted into the axon. Direct sorting and axonal transport from the TGN is presumed to be a major route for axonal membrane proteins (in analogy with the epithelial model system), but direct experimental evidence is scarce. Some somatodendritic proteins have in fact been shown to travel in distinct carriers from axonal proteins. For instance, vesicles containing the somatodendritic transferrin receptor are not seen to enter axons and only move in dendrites (Burack et al. 2000) whereas vesicles containing the axonal cell adhesion molecule L1/NgCAM are found to travel in both axons and dendrites. Whether these vesicular carriers were endosomal or TGN-derived was not established. The best evidence currently for direct transport from the TGN to the axon is a live imaging study from the Hirokawa group, showing preferential axonal transport of vesicles containing VSV-G (an axonal variant) and amyloid precursor protein βAPP after release from a biosynthetic block in the ER/Golgi using low temperature or Brefeldin A (Nakata and Hirokawa 2003).

Multiple studies have shown that, similarly to epithelial cells, neurons are capable of generating multiple vesicular or tubular carriers containing biosynthetic cargos, although the exact identity and compartment of origin for these carriers has not been established. There are at least two classes of vesicles/tubules that carry distinct axonal cargos (Kaether et al. 2000). Multiple axonal cargos can also share the same carrier (Nakata and Hirokawa 2003), making it likely that each cargo does not have a dedicated vesicular carrier, but cargos with similar destinations can be cotransported.

Some cargos, such as the adhesion receptor L1/NgCAM (Yap et al. 2008b), travel to the axon in carriers derived from somatic endosomes rather than from the TGN (see "Transcytosis").

Indirect Polarized Delivery by Transcytosis

In epithelia, endocytosis does not always result in recycling back to the plasma membrane domain of origin, but rather to the opposite domain, a process termed transcytosis. Transcytosis can occur both from the apical to basolateral domain, and vice versa. Typically, it is a property associated with specific receptors that function in the transcellular transport of specific cargo, such as the transfer of IgA or IgM by the polymeric immunoglobulin receptor (Mostov et al. 1995). In some epithelia, however, transcytosis is a more general pathway. In hepatocytes, for example, the large majority of membrane proteins that appear at the apical (bile canalicular) surface are first inserted at the basolateral (sinusoidal) surface, internalized, and then sorted into apically directed transcytotic vesicles at the level of recycling endosomes (Sheff et al. 1999; Tuma and Hubbard 2003). In neurons, transcytosis provides an axonal delivery pathway for at least some axonal membrane proteins, such as L1/NgCAM (Wisco et al. 2003): Newly synthesized L1/NgCAM is first delivered to the somatodendritic domain, followed by endocytosis and transport to the axon. Other proteins might also use transcytosis for axonal delivery, such as cannabinoid receptor CB1R (Leterrier et al. 2006), Caspr2 (Bel et al. 2009), and TrkA (Ascano et al. 2009).

One molecular regulator on the trancytotic pathway is the neuronal-specific endosomal protein NEEP21 (neuron-enriched endosomal protein of 21 kD): Down-regulation of NEEP21 levels results in missorting of endocytosed NgCAM to the somatodendritic domain and to lysosomes (Yap et al. 2008b). The endosomal route taken by L1/NgCAM to the axon is diagrammed in Figure 1.

TGN-based sorting also contributes to L1/NgCAM polarity. L1/NgCAM contains a specific tyrosine-based motif (YRSLE) in the cytoplasmic tail that mediates sorting from the TGN to the somatodendritic domain (Yap et al. 2008a). Interestingly, NgCAM accumulates on the apical domain in MDCK cells and reaches its apical destination via transcytosis, on a pathway apparently very similar to the one in neurons (Anderson et al. 2005). The same tyrosine-based motif also mediates the initial basolateral sorting of NgCAM in MDCK cells. In MDCK cells transcytotic trafficking of NgCAM is regulated by phosphorylation (Anderson et al. 2005): the epithelial-adaptor AP-1B recognizes the YRSLE signal and mediates basolateral sorting of newly-synthesized NgCAM. The YRSLE motif is also recognized by the endocytosis clathrin adaptor AP-2, which mediates endocytosis of NgCAM into basolateral endosomes. The YRSLE motif is then subject to phosphorylation, likely by a src family kinase. This phosphorylation inactivates the basolateral signal and inhibits AP-1B binding. NgCAM with a phosphorylated YRSLE motif is therefore not recycled back to the basolateral domain, but rather transcytosed to the apical domain based on apical sorting signals. The apical signals are only active when the YRSLE basolateral signal is inactivated by phosphorylation (see Fig. 2). In agreement with this mechanism, NgCAM carrying a point mutation in the YRSLE motif (NgCAM Y33A) is unable to undergo transcytosis in neurons and travels to the axon on a direct, endocytosis-independent pathway instead (Wisco et al. 2003). A similar mechanism involving spatial regulation of adaptor binding via phosphorylation thus likely also regulates NgCAM transcytosis in neurons, with the difference that an adaptor other than AP-1B likely mediates somatodendritic sorting.

In addition to the YRSLE somatodendritic signal, an axonal signal maps to a 15 amino acid gycine- and serine-rich stretch in the cytoplasmic tail of L1/NgCAM. Both signals are required for the sequential regulated routing via the multi-step transcytotic pathway (Yap et al. 2008a). Surprisingly, L1/NgCAM contains a second sufficient axonal targeting signal in the extracellular domain (Sampo et al. 2003). It is not clear why L1/NgCAM contains two sufficient axonal signals, but the presence of the second signal improves axonal targeting.

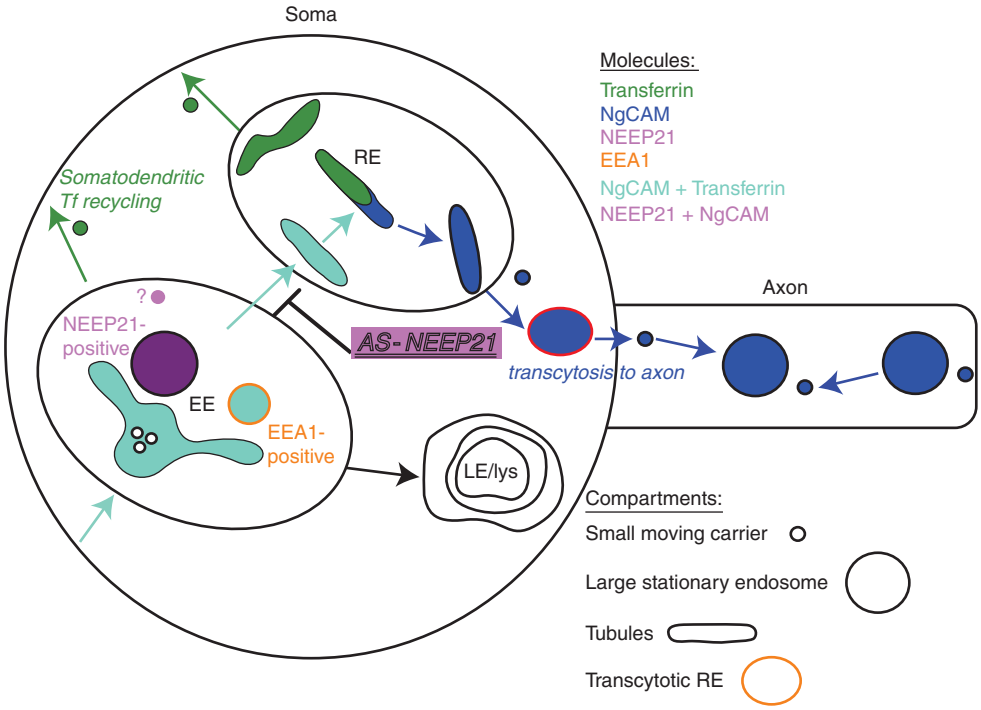

Figure 1. Endosomal compartments involved in NgCAM transcytosis to the axon. NgCAM is endocytosed into EEA1-containing early endosomes with transferrin. Transferrin (Tf) recycles from the early endosome whereas NgCAM traverses NEEP21-positive early endosomes and reaches recycling endosomes. Transferrin is also found in recycling endosomes. In recycling endosomes, transferrin and NgCAM occupy partially overlapping domains and are ultimately sorted away from each other. NgCAM then enters axons in small motile endosomally derived carriers and is transported anterogradely via fast axonal transport. Stationary endosomes containing NgCAM along axons provide stations from which small carriers can likely bud or fuse.

The reason why NgCAM transcytoses to the axon is not immediately apparent, and many questions remain regarding a possible biological function of this circuitous targeting route. For both CB1R (Leterrier et al. 2006) and TrkA (Ascano et al. 2009), ligand binding drives the transcytotic pathway, raising the possibility that routing of axonal proteins is subject to ligand-mediated signaling. It is possible that the transient appearance of L1/NgCAM on the somatodendritic surface also serves a purpose, such as signaling in dendrites, binding of a ligand, or a guidance or feedback function that coordinates dendrite and axonal growth. Given the fact that the YRSLE motif is subject to regulation by phosphorylation (Schaefer et al. 2002), it is easily conceivable that transcytotic routing can be turned on and off by the cell in a signal cascade-dependent manner. To what ultimate end is an intriguing question to be pursued.

Axonal Targeting by Selective Endocytosis/ Retention

For several axonal proteins axonal accumulation is dependent on endocytosis and does not occur by preferential sorting from the TGN. Rather, these axonal proteins are first inserted in a signal-independent fashion into both somatodendritic and axonal domains, and subsequently the somatodendritic (i.e., missorted) pool of receptor is removed by preferential endocytosis coupled to specific retention/anchoring in the correct axonal domain. Presumably, the endocytosed misplaced receptor

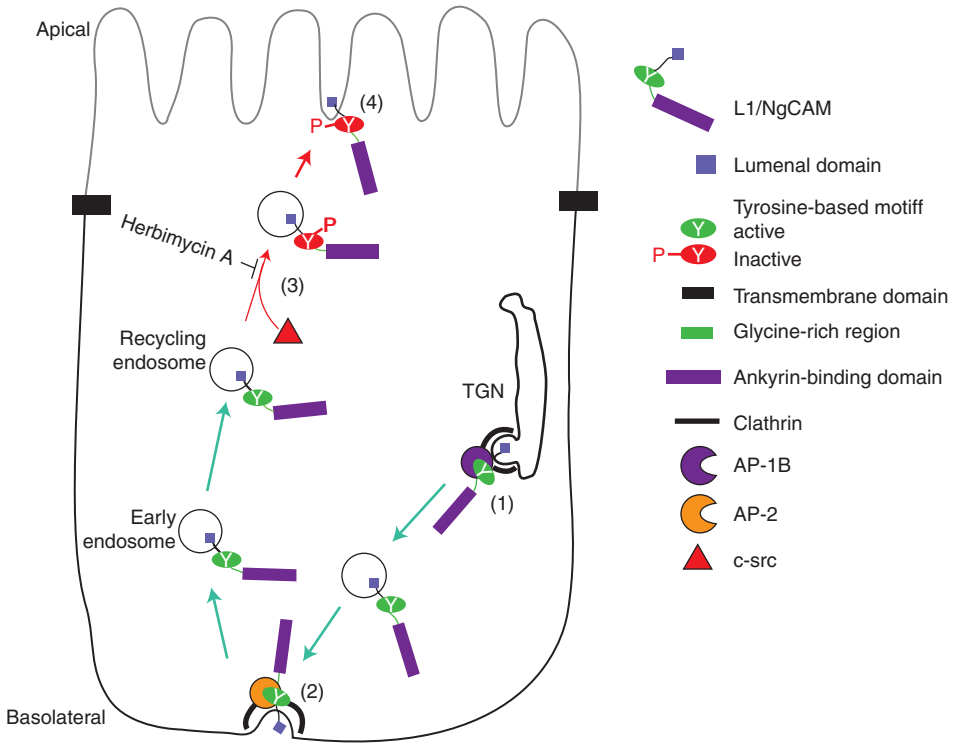

Figure 2. Regulation of NgCAM transcytotic routing by spatial regulation of phosphorylation of the adaptor binding site (based on Anderson et al. 2005). NgCAM is sorted to the basolateral domain based on a tyrosine-based motif (YRSLE) in its cytoplasmic tail. This signal is recognized by the basolateral sorting adaptor AP1B. The site of action of AP1B-based sorting is either the TGN or the recycling endosome. NgCAM is then exocytosed on the basolateral plasma membrane. The clathrin adaptor AP2 binds to the YRSLE motif and mediates endocytosis into endosomes. At some point after endocytosis, the YRSLE is phosphorylated by a src family kinase. This phosphorylation event prevents binding of AP1B and thereby redirects the endocytosed NgCAM away from basolateral recycling into an apical-directed transcytotic route. In the presence of the kinase inhibitor herbimycin A, NgCAM accumulates on the basolateral, instead of the apical, domain. An analogous mechanism likely operates for NgCAM in neurons.

pool is subsequently degraded in lysosomes, but this has not been shown experimentally. Axonal targeting has only been studied for a small number of axonal proteins and selective endocytosis/retention is the most frequent pathway described to date (Fache et al. 2004; Garrido et al. 2003; Sampo et al. 2003; Xu et al. 2006). This pathway contrasts with transcytosis in that transcytosing cargo is not primarily degraded but rather recycled to the axonal surface. Because both the selective endocytosis/retention pathway and transcytosis are dependent on endocytosis, distinguishing the two pathways requires careful experimentation. No guidance receptors have been found to be targeted to axons via selective endocytosis/retention, but few have been studied at all, so it might just be a matter of systematically investigating the endocytosis dependence of guidance receptors to find examples. Long-range axonal targeting thus relies on TGN-based sorting via poorly-understood signals and machinery (including adaptors and motors), endocytic removal of misplaced proteins, and either degradation or signal-mediated recycling to the axon from somatodendritic endosomes. The relative contribution of each of these mechanisms for any particular guidance receptors

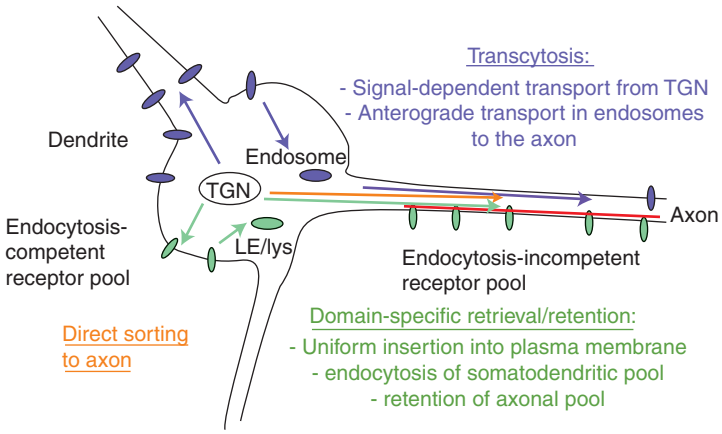

Figure 3. Three axonal transport pathways. Transmembrane receptors can accumulate on the axonal surface by three mechanisms:

1. They can be directly sorted to the axon from the TGN based on sorting signals and association with axonally directed motor proteins (orange pathway: direct sorting).
2. They can be sorted in the TGN to the somatodendritic domain and be redirected to the axon after endocytosis (purple pathway: transcytosis).
3. They can be uniformly inserted into the plasma membrane in the axonal and somatodendritic domains and then be removed preferentially from the somatodendritic domain and stabilized in the axon (green pathway: domain-specific retrieval/retention).

will need to be investigated for each case. The three pathways are depicted in Figure 3.

Regulation of Diffusibility: Maintaining Adhesion Receptors in Axons

Once membrane proteins are inserted into the plasma membrane, they can diffuse and any enrichment in one or another domain could be lost because of free lateral diffusion. Diffusion of receptors can be slowed or brought to a virtual hold by several mechanisms: binding of the extracellular domain to immobilized extracellular matrix, binding of the cytoplasmic tail to immobilized cytoskeletal elements, clustering of receptors in lipid raft domains, and passive restriction of diffusion because of the presence of clustered immobile obstacles ("percolation diffusion"/"picket fence"). Some of these mechanisms rely on specific protein sequences or posttranslational modifications of the receptor to mediate binding interactions with specific extracellular or cytoskeletal components. Receptors can also be more generally restricted in their diffusibility by features of the membrane that affect all membrane-resident receptors regardless of their sequence. Such signal-independent mechanisms include confined modes of diffusion (reviewed in Choquet and Triller 2003; Kusumi et al. 2005; Newpher and Ehlers 2008). The main molecular explanations for confined diffusion are "corralled diffusion" (because of the spectrin-based cytoskeleton) and "percolation diffusion" (because of a large number of immobilized proteins in the membrane). All of these types of diffusion restriction operate in neurons.

A Membrane Diffusion Barrier in the Axon Initial Segment

In neurons, a barrier to diffusion exists at the axon initial segment (AIS) that prevents (or slows) intermixing of axonal membrane proteins with somatodendritic ones: several membrane proteins (including L1) are largely freely diffusible on the more distal axon of a cultured

neuron, but their diffusion is highly restricted in the AIS (Winckler et al. 1999). The diffusion barrier is disrupted by drugs that depolymerize actin filaments (Nakada et al. 2003; Winckler et al. 1999) and is deficient in neurons derived from βIV-spectrin deficient mice (Nishimura et al. 2007) and in ankG-deficient neurons (Song et al. 2009). The diffusion barrier at the AIS is therefore likely due to the ankyrin/spectrin-based creation of a dense obstacle course of tethered, immobilized ankyrin-binding proteins at the AIS, such as voltage-gated sodium channels. Restricted diffusion in the AIS is also observed for lipids (Nakada et al. 2003) and for the GPI-linked protein Thy1 (Winckler et al. 1999), suggesting that the diffusion barrier in the AIS not only affects proteins that can directly bind to ankyrin, but also restricts diffusion more generally by creating corrals and picket fences based on actin/spectrin/ankyrin meshworks.

Cytoplasmic Diffusion Barrier in the Axon Initial Segment

Recently, a barrier to the diffusion of cytoplasmic components in the axon was reported (Song et al. 2009). This cytoplasmic filter operates in the same location, i.e., the axon initial segment, is elaborated at the same time point in development in cultured neurons, and requires similar molecular components, i.e., F-actin and ankyrinG, as the membrane diffusion barrier described earlier. The cytoplasmic filter impedes the entry of large, but not small dextrans into the axon. Larger particles (such as vesicles) can only pass through the cytoplasmic filter if powered by a highly efficient microtubule motor such as KIF5. Cargos transported by the slower motor KIF17 (which usually transports somatodendritic cargos in dendrites) are still impeded by the cytoplasmic filter. Interestingly, features of both the motor and the cargo itself determine the entry rate through the cytoplasmic filter. The cytoplasmic filter is actin/ankyrin/spectrin-based and the mesh created impedes in a nonspecific way the entry of particles past a certain size limit. Entry is made possible only to particles that have the ability to attach to a subset of efficient microtubule motors, which then specifically attach to microtubules to gain passage through the filter. What exact molecular features of motor, cargo, and microtubules are crucial to controlling entry into the proximal axon is still to be discovered. The importance of maintaining functional AIS filters is highlighted by the finding that the axons of neurons with down-regulated ankyrinG slowly acquire dendritic features, such as MAP2 and dendritic spines (Hedstrom et al. 2008).

"X Marks the Spot" – An Instructive Role for the Axon Initial Segment

In epithelial cells, polarity has been proposed to be generated by the hierarchical interplay of three molecular macroassemblies (Mellman and Nelson, 2008). At the highest level of control, local extracellular cues (level 1) cause the recruitment of cytoplasmic polarity machinery (level 2) to the plasma membrane to "mark the spot" for fusion of vesicles, which contain cargos sorted at the TGN (level 3). Cargo-containing vesicles can thereby only fuse in the correct domain because the required fusion machinery is assembled there in response to extracellular cues. Could a similar hierarchical principle also underlie polarized secretion in neurons? Most likely yes, but an additional level of control operates in neurons at the level of the axon initial segment. Data discussed earlier indicate that entry into the axon is restricted both in the cytoplasm and at the plasma membrane. Vesicles carrying the somatodendritic transferrin receptor are not seen to enter the axon by live imaging (Burack et al. 2000). This is presumably a reflection of the cytosolic barrier, and the inability of the transferrin receptor-containing vesicles to attach to the appropriate microtubule motor. In contrast, vesicles containing certain axonal cargos (such as βAPP) are seen to preferentially transport along microtubules that extend into axons when they bud from the somatic TGN (Nakata and Hirokawa 2003).

There are clear indications that the axonal microtubules are distinct from dendritic microtubules and might allow translocation of only

certain KIFs. For instance, distinct and largely nonoverlapping sets of microtubule-associated proteins are found enriched on axonal and dendritic microtubules (Caceres et al. 1986). MAP2 is largely dendritic and tau axonal. The type of MAP decorating the microtubules might thus bias the sets of KIF motors that will use them. Such evidence certainly exists in vitro (Dixit et al. 2008; Marx et al. 2006). In addition, microtubules carrying posttranslational modifications, such as tyrosination/detyrosination, show preferential interactions with certain KIFs. KIF5C, for example, preferentially binds detyrosinated and acetylated microtubules (Dunn et al. 2008). It was shown recently in fact that loss of the tubulin tyrosinase enzyme causes loss of neuronal polarity with many neurons elaborating multiple tau-positive processes (Konishi and Setou 2009). Because the somatodendritic domain has a high ratio of tyrosinated to detyrosinated microtubules whereas the axon initial segment and the axon have a low ratio, KIF5 binding and movement are favored on the detryosinated microtubules found preferentially in the axon. Neurons therefore not only "mark the spot" for membrane fusion by locally assembling polarity complexes at the final target membrane (a role for par3/6-KIF3 complex has been proposed (Nishimura et al. 2004)), but additionally bias the transport of vesicles by favoring the entry of axonal vesicles into the axonal initial segment. This additional level of control in neurons might prevent the futile large-scale motoring of incorrect vesicles to far-away axonal growth cones.

LOCAL CONTROL OF GUIDANCE RECEPTOR DISTRIBUTION AT GROWTH CONES

The location, levels, and residence time of adhesion and guidance receptors crucially influence their functional activity (Long and Lemmon 2000) and hence axon guidance and growth. This is similar to cell migration in which it is thought that targeted recruitment of adhesion molecules, like integrins, to the cell's leading edge promotes directional movement of the cell (Bretscher and Aguado-Velasco 1998). Similarly recruitment of L1 to the edge of the growth cone powers L1-mediated growth cone advance (Kamiguchi and Lemmon 2000). In neurons, many secretion events are not constitutive, but regulated by extracellular signals or electrical activity. Regulated fusion is likely an important mechanism for locally controlling the levels of guidance receptors in growth cones. Similarly, regulated endocytic removal of guidance receptors can rapidly and dynamically change the local levels of guidance receptors. The same three mechanisms discussed for the regulation of long range biosynthetic trafficking (regulation of insertion, diffusibility, and removal) are thus in effect locally at growth cones and at sites of synaptogenesis to change the levels and distribution of receptors. We are only at the beginning of unraveling how guidance cues and signaling through guidance receptors and through other pathways regulate insertion, diffusion, and removal of guidance receptors locally. Again, we will focus on L1/NgCAM as an example of these local mechanisms. Work in several laboratories over the years has contributed to our understanding of the molecular mechanisms underlying L1-mediated axon growth. Local insertion, local endocytosis and recycling, as well as regulated binding to mobile and immobile cytoskeletal elements are all involved in L1-mediated outgrowth and growth cone steering (Fig. 4).

Regulation of Local Insertion

L1 is a homophilic cell adhesion molecule, but also has heterophilic binding partners (Maness and Schachner 2007). There is good evidence that L1 ligation leads to stimulation of local fusion of vesicles preferentially at the site of ligand contact. If large beads coated with L1-Fc are placed into contact with growth cones on one side, L1 accumulates at the site of contact (Alberts et al. 2003; Dequidt et al. 2007). Live imaging shows that some of the initial accumulation is caused by the increased directional trafficking of vesicles to the bead contact. Lateral diffusion of receptors already at the cell surface also contributes (Dequidt et al. 2007; see the following). The exact nature of the vesicles

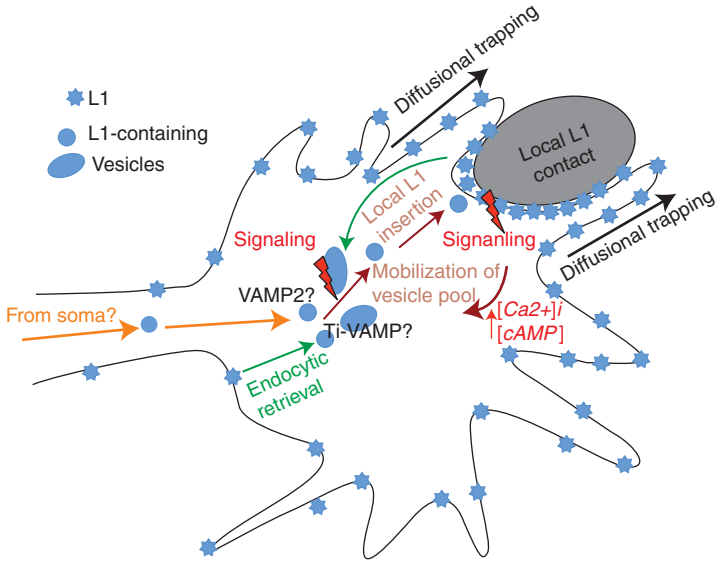

Figure 4. Local regulation of L1 surface levels at the growth cone. L1 ligation via L1 homophilic binding leads to diffusional trapping of surface L1 at the contact site and mobilization and exocytosis of L1 from vesicular pools. Endocytic retrieval from the rear of the growth cone maintains a gradient of L1 across the growth cone and provides a pool of vesicular L1 available to be exocytosed locally.

prompted to fuse locally is not known, but at least some of them are endosomally derived. Both the toxin-insensitive VAMP (TI-VAMP/VAMP7) (Alberts et al. 2003), and VAMP2 (Tojima et al. 2007) have been implicated in the local fusion reaction and might act on distinct vesicular pools. No such local fusion or accumulation of L1 are induced by beads coated with N-cadherin (Dequidt et al. 2007). The exact signaling pathway downstream of L1 ligation to result in local fusion are still being elucidated, but local increases in cAMP and intracellular calcium from intracellular stores are required (Tojima et al. 2007). The locally stimulated fusion events lead to local expansion of the growth cone membrane and contribute to directed growth cone turning.

Local regulation of vesicle trafficking and fusion has also been reported in the case of the adhesion molecule NCAM. Plasma membrane-resident NCAM can tether TGN-derived vesicles and cause local accumulation of TGN-derived vesicles at sites of homophilic NCAM interactions (Sytnyk et al. 2002). These vesicles might subsequently fuse and contribute to building a new synapse at the site of the initial NCAM-mediated contact. Future sites of new synaptogenesis can therefore locally regulate the transport speeds, halt times, and probably the fusion likelihood of vesicles. This kind of local signaling contributes to the accumulation of presynaptic components that build the presynaptic active zone (Ahmari et al. 2000; Waites et al. 2005). The details of the signaling events downstream of ligation will be important topics of future inquiry.

Regulation of Local Endocytosis

It has long been known that local endocytosis is required for L1-mediated growth cone advance (Kamiguchi and Lemmon 2000). L1-mediated growth cone advance occurs when L1 engages in homophilic binding at the growth cone edge (P domain) and engages retrograde actin flow to advance the growth cone (Gil et al. 2003). When L1 reaches the central portion of the growth cone (C domain), it is endocytosed, and signals from endosomes through the MAP kinase (and possibly other) pathways (Kamiguchi

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and Lemmon 2000; Schaefer et al. 1999). Inhibition of endocytosis or of MAP kinase signaling impairs L1-mediated outgrowth. L1 endocytosis is substrate-dependent and only occurs on L1 as a consequence of homophilic binding. Furthermore, a gradient of L1 on the growth cone is required for axon L1-mediated outgrowth. This gradient is established by endocytosis (Kamiguchi and Yoshihara 2001).

As discussed earlier, the cytoplasmic tail of L1 contains a tyrosine-based endocytosis signal (YRSLE) that mediates binding to the clathrin endocytosis adaptor AP-2 (Kamiguchi et al. 1998b). Binding to AP-2 is decreased upon phosphorylation of the endocytosis motif downstream of src signaling (Schaefer et al. 2002). This may also affect transcytosis from the soma. L1 endocytosis is therefore regulated by kinase cascades, and thus subject to regulation by local signaling events. Interestingly, L1 ligation causes dephosphorylation of the L1 endocytosis motif and triggers endocytosis (Schaefer et al. 2002). The dynamic nature of L1-L1 interactions has been studied in detail using sophisticated live imaging and quantitative tracking approaches (Dequidt et al. 2007). In this study, L1 molecules accumulated at an L1-bead contact endocytose rapidly and exchange with intracellular pools. The dynamic behavior of L1 at a homophilic bead contact is strikingly different from the more static behaviors of N-cadherin, TAG1, or NrCAM at homophilic bead contacts, all of which make stable adhesions with little turnover (Falk et al. 2004; Thoumine et al. 2006). How the highly dynamic nature of L1 contacts affects the functions of L1 in outgrowth and pathfinding remains to be determined (Thoumine 2008).

L1 is also engaged in a large number of heterophilic binding interactions, both in *cis* and *in trans* (Maness and Schachner 2007). For some of these interactions L1 endocytosis has been shown to be important. For example, L1 is required for Sema3A-mediated growth cone collapse. L1 endocytosis is involved in downregulating the levels of the semaphorin3A coreceptor, neuropilin-1 (Bechara et al. 2007). The ability of L1 to bind ERM proteins via its cytoplasmic tail is important in these semaphorin-mediated events (Mintz et al. 2008). The endocytosis of the L1-neuropilin-1 complex also leads to local signaling and disassembly of focal adhesions (Bechara et al. 2008). Endocytosis, signaling, and subsequent disassembly of focal adhesions therefore might lead to growth cone collapse downstream of Sema3A.

Sema3D is another repulsive guidance molecule that acts—at least in part—by regulating surface levels of L1. Work in zebrafish shows that Sema3D overexpression increases the surface levels of L1, leading to increased fasciculation of axons (Wolman et al. 2007). The exact signaling cascades connecting sema3D and L1 surface levels are not yet known, but membrane traffic is a potential candidate for regulation.

It is clear that removal of a receptor from the surface is not the only consequence of endocytosis, but rather elaborate signaling cascades are activated downstream of endocytosis (Miaczynska et al. 2004). Little is known about the endosomes from which adhesion receptors, such as L1, signal. For other endocytosing receptors, we know that signaling outcomes can differ in important ways depending on the endocytic route taken (clathrin-dependent or -independent) and the subsequent postendocytic trafficking (recycling endosome, late endosome, etc.) (for examples see Deinhardt et al. 2007; Le Roy and Wrana 2005). Furthermore, the residence time in these signaling endosomes can also be regulated and greatly influence signaling outcomes (Zoncu et al. 2009). These events downstream of the endocytosis event per se thus will be important topics of study in the future.

Regulation of Local Diffusibility

The local concentration of guidance receptors is also influenced by the degree to which they can diffuse in the plasma membrane. Live imaging of L1-GFP at L1 bead contacts shows that rapid diffusion of L1 contributes to the accumulation of L1 at a new bead site (Dequidt et al. 2007). Local insertions (discussed earlier) are additionally responsible for L1 accumulation at young (but not mature) contacts. Many

receptors, including L1, can bind cytoskeletal elements and thereby be locally immobilized. L1 binds both directly to ankyrin via the an ankyrin binding motif (Davis and Bennett 1994) and indirectly to actin via ERM proteins (Dickson et al. 2002) via a membrane-near binding site (Sakurai et al. 2008). In fact, mice carrying a knock-in of ankyrin-binding deficient L1 (L1 Y1229H) show axon guidance defects of retinal ganglion cell axons (Buhusi et al. 2008). Both of these binding interactions are subject to regulation by phosphorylation: ankyrin-binding is inhibited by phosphorylation of the ankyrin binding site downstream of MAP kinase signaling (Whittard et al. 2006) and ERM binding is inhibited by phosphorylation of its binding site downstream of src family kinase signaling (Sakurai et al. 2008). Binding to ankyrin and ERM might be mutually exclusive. When L1 is bound to ankyrin, it shows little diffusion in the plasma membrane (Garver et al. 1997). If it is not bound to ankyrin, it can bind to retrograde actin flow and participate in powering growth cone advance (Gil et al. 2003). L1 bound to ankyrin is likely not available for endocytosis. Furthermore, signaling cascades downstream of L1 ligation are modulated in complex and incompletely understood ways. For instance, ankyrinB modulates levels of cAMP in neurons growing on L1 (Ooashi and Kamiguchi 2009). The coordinated interplay of local insertion, local diffusibility, and local endocytosis thus regulates the precise spatiotemporal distribution of L1 at the advancing growth cone (Fig. 4) and thereby the spatial profile of signaling cascades that lead to changes in cytoskeletal arrangements and membrane trafficking to result in growth cone turning.

OUTLOOK FOR OTHER GUIDANCE RECEPTORS

The trafficking of guidance receptors is crucial to the correct wiring of the brain. We are only at the beginning of understanding the multiple ways in which guidance receptor distribution is determined: long distance trafficking from the soma as well as local mechanisms such as endocytic removal, recycling, and retention by diffusional restriction coordinately set the spatial parameters of guidance receptor distribution. The details of the regulation of the global and local mechanisms still await discovery for most guidance receptors. Whereas this article focused on describing the conceptual advances made in the field of receptor trafficking using L1 as a well-studied example, the importance of regulating trafficking is also beginning to emerge for many other guidance receptors. We refer the reader to other relevant articles, including those on receptors for Ephrins/Eph, netrins (DCC and UNC5 receptors), and semaphorins (Neuropilins and Plexins), and on Robo receptors.

In both *Drosophila* and vertebrates, Robo receptors provide a particularly striking example of compartmentalized localization where the control of intracellular traffic may play a key role in regulating Robo surface expression. The midline is an important intermediate target where in-growing axons growth cones make precise targeting decisions (see Dickson and Zou 2010). The repellent protein slit is expressed at the midline and acts on Robo receptors to repel growth cones from crossing or recrossing the midline. Because all axons express Robo, it was unclear how commissural axons expressing Robo initially overcame the slit-mediated repulsion. Much work revealed a complex and fascinating mechanism by which trafficking of Robo to the growth cone is controlled by the Commissureless (Comm) protein (Keleman et al. 2002; Keleman et al. 2005; Myat et al. 2002): in the presence of Comm, Robo is directed to endosomes instead of the plasma membrane, presumably preventing its ability to interact with slit.

In vertebrates, Robo and slit receptors also regulate crossing of commissural axons in the developing spinal cord. Because vertebrate genomes lack a recognizable Comm homolog, other mechanisms are likely involved in opposing the repulsive action of slit at the midline. Work in the Tessier-Lavigne lab has revealed that Robo 1 and 2 localize most highly to the postcrossing portion of commissural axon in the developing spinal cord and play crucial roles

in regulating growth cone guidance at the midline in mice (Long et al. 2004). Robo3, on the other hand, behaved quite differently. Robo3 is expressed as two splice variants (Robo3.1 and Robo3.2) that localize distinctly (Chen et al. 2008): Robo3.1 is expressed highly on the precrossing segment of commissural axons whereas Robo3.2 is expressed highly on the postcrossing segment. The localization of these Robo isoforms reflects their role in guidance: Robo3.1 favors crossing by silencing slit repulsion whereas Robo3.2 blocks crossing. Regulating exocytosis, endocytosis, and diffusion of these receptors could contribute to the observed restricted surface distribution, especially because the alternative splicing creates different cytoplasmic domains. Different receptors, and even splice isoforms of the same receptor (e.g., IgG Fc receptors; Miettinen et al. 1989) can show entirely distinct capacities for internalization. However, the mechanism underlying the differential localization of the two Robo isoforms is not yet understood.

A recent paper in *Drosophila* may shed some light on how neurons restrict the distribution of Robos as well as a second guidance receptor, derailed (DRL). Hiromi and colleagues (Katsuki et al. 2009) show that Robo 2 and 3 are enriched on the distal axon segments whereas DRL is enriched on the proximal segment. This differential distribution is maintained when *Drosophila* neurons are cultured in isolation and therefore reflects a cell-autonomous mechanism. This is reminiscent of the compartmentalized localization of many receptors in cultured vertebrate neurons, an observation made many years ago by Gary Banker and others (Craig and Banker 1994; Lai and Jan 2006). Hiromi and colleagues show that inhibition of endocytosis leads to loss of compartmentalized distribution of DRL, but the distal localization of Robo3 was only mildly affected. As discussed in more detail earlier, several axonally localized receptors in cultured vertebrate neurons were previously shown to be dependent on endocytosis for proper localization as well (reviewed in Lasiecka et al. 2009). In addition to endocytosis, however, Hiromi and colleagues show that the diffusion of membrane receptors is greatly slowed across the compartment boundary that delineates the proximal and distal axon segments. Whether this diffusion restriction is molecularly similar to the actin/ankyrin/spectrin based diffusion barrier at vertebrate axon initial segments (Arnold 2009; Boiko and Winckler 2003) remains to be determined, but clearly evolutionarily conserved mechanisms for restricting the surface distribution of membrane receptors exist and it will be imperative to understand the regulation for each class of guidance molecule. Furthermore, the complex crosstalk of trafficking with signaling pathways and cytoskeletal arrangements needs to be elucidated to gain a complete understanding of how the brain wires itself into functional units.

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