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Membrane traffic in polarized neurons

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Abstract

The plasma membrane of neurons can be divided into two domains, the soma-dendritic and the axonal. These domains perform different functions: the dendritic surface receives and processes information while the axonal surface is specialized for the rapid transmission of electrical impulses. This functional specialization is generated by sorting and anchoring mechanisms that guarantee the correct delivery and retention of specific membrane proteins. Our understanding of neuronal membrane protein sorting is primarily based on studies of protein overexpression in cultured neurons. These studies revealed that newly synthesized membrane proteins are segregated in the Golgi apparatus in the cell body from where they are transported to the axonal or dendritic surface. Such segregation presumably depends on sorting motifs in the proteins' primary structure. They appear to be located in the cytoplasmic tail for dendritic proteins and in the transmembrane-ectodomain for axonal proteins. Recent studies on neurotransmitter segregation suggest that anchoring in the correct subdomain of the plasma membrane also requires cytoplasmic tail information for binding to the cytoskeleton either directly or by linker proteins. Both mechanisms, sorting and retention, gradually mature during neural development. Young neurons appear to develop initial polarity by other mechanisms, presumably analogous to the mechanisms used by migrating cells.

Keywords: Neuronal polarity; Axon; Dendrite; Sorting; Retention; *trans*-Golgi network

1. Introduction

The transmission of a signal in a neuron is in principle divided into four phases: (1) signal input, (2) signal integration, (3) signal conduction, and (4) signal output [1]. To achieve directionality each of the phases are spatially segregated within the neuron, as are the proteins on which the specific phases are based. To highlight the correlation between function and protein distribution we briefly outline the transmission of a signal and mention some of the important proteins (see Fig. 1). *Signal input*: neurotransmitter (NT) receptors specific for the NT released from the presynaptic site are concentrated at the postsynaptic site [2,3]. The receptors, which can be

Abbreviations: AchR, acetylcholine receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazol propionate; APP, amyloid precursor protein; CT, cytoplasmic tail; Dlg-A, disc large; FPV, fowl plague virus; GABA, γ-aminobutyric acid; GluR1, AMPA subclass glutamate receptor 1 subunit; GlyR, glycine receptor; GPI, glycosylphosphatidylinositol; HA, hemagglutinin; LDL, low density lipoprotein; MDCK, Madin-Darby canine kidney; nAchR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartate; NT, neurotransmitter; PDZ, PSD-95, Dlg-A, ZO-1; pIgR, polyimmunoglobulin receptor; PSD, postsynaptic density; SFV, Semliki Forest virus; SH3, src homology 3; TGN, *trans*-Golgi network; TfR, transferrin receptor; VSV, vesicular stomatitis virus; ZO-1, zonula occludens-1

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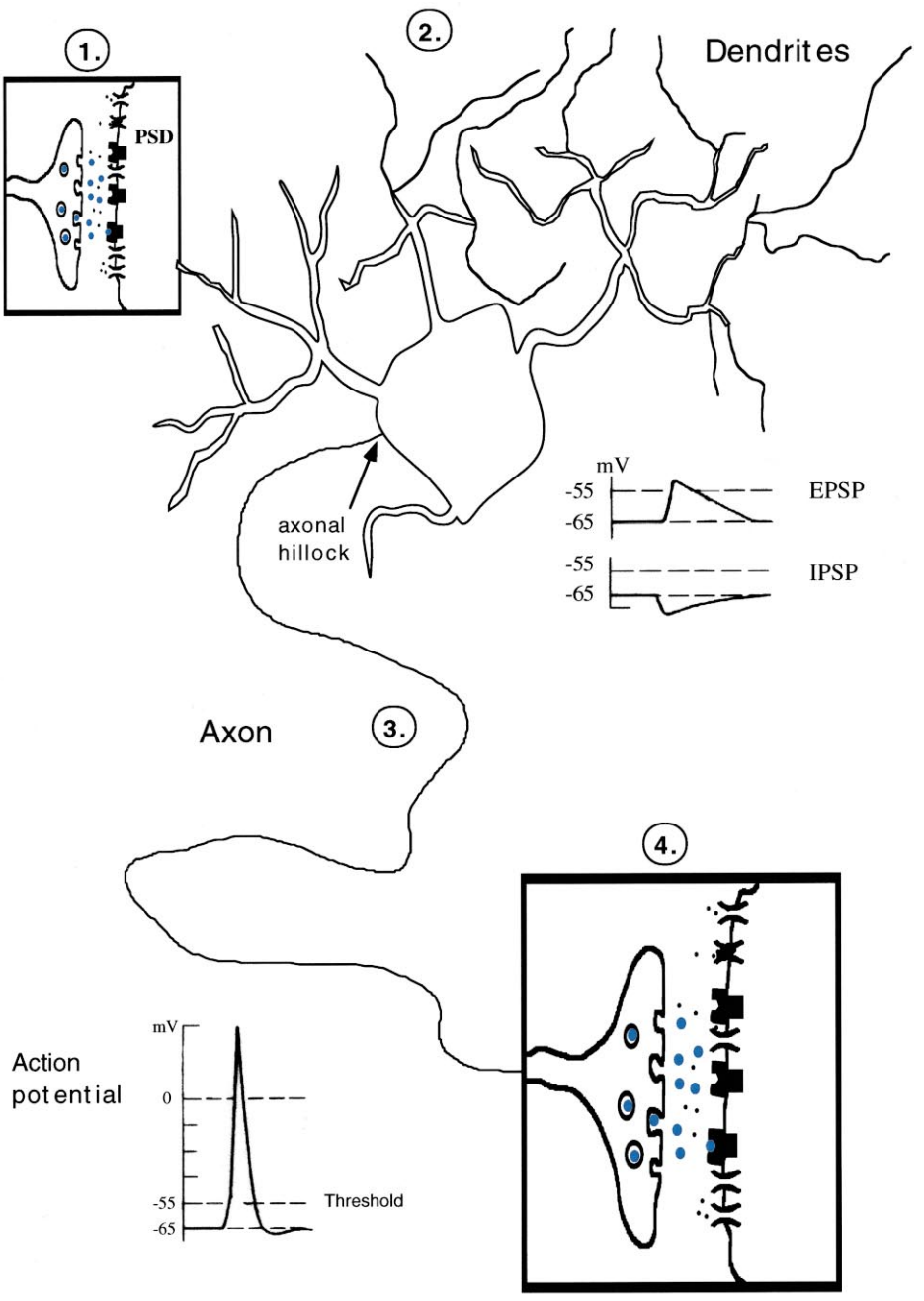

Fig. 1. Basic signal propagation in neurons. (1) Released neurotransmitter molecules bind to their specific receptors located in the postsynaptic membrane inducing the opening of ion channels and, hence, a change in the membrane potential in the form of a postsynaptic potential, either excitatory (EPSP) or inhibitory (IPSP). (2) The different stimuli become integrated on the dendrites and the cell body. At the axonal hillock an action potential is formed if the postsynaptic potential depolarizes the membrane over a certain threshold. (3) The action potential travels actively down the axon. (4) At the nerve terminals membrane depolarization leads to the exocytosis of neurotransmitter. For proper functioning of these steps the specifically involved proteins have to be localized to their place of action (see text).

ionotropic or coupled to second messengers, modulate the chemical signal into an electrical signal by allowing ion flow across the plasma membrane upon binding of the NT. *Signal integration*: the electrical signals generated at the dendritic terminals are then integrated via the dendrites and the cell body. Until

recently, the propagation of the signal in this phase was thought to be passive, i.e. based on the isolating properties of the plasma membrane and independent of the opening of voltage gated ion channels. However, calcium mediated action potentials occur in dendrites [4] and voltage sensitive N-type calcium channels are also localized to dendrites [5] suggesting modulation of the 'passively' propagating signal [4]. (Note that there are also actively back-propagating action potentials found in dendrites [131].) *Signal conduction*: at the point of the axon hillock the integrated, graded signal triggers an action potential in an all or none fashion. Upon local depolarization of the membrane voltage dependent sodium channels open and allow adjacent parts of the plasma membrane to depolarize which again causes voltage gated sodium channels to open. Hence, the action potential propagates actively down the whole axon. Voltage gated sodium channels are mainly found at the axon hillock but also to a lesser extent at the cell body and very proximal dendrites [6,7]. This correlates with the electrophysiological finding that the threshold to induce an action potential is lowest at the axonal hillock [8]. *Signal output*: when the action potential reaches the synapses at the ending of the axon, voltage gated calcium channels open and the increased calcium concentration triggers vesicle fusion and NT release [9]; the electrical signal is remodulated into a chemical signal. All the described events are based on the proper position of membrane proteins to different membrane domains. Furthermore, even members of the same family are localized to different domains within one cell as in case of the N-type calcium channels [5] and the voltage gated sodium channels [10]. The picture becomes even more complex when other subdomains of the neuronal membrane are taken into account. Synapses occur not only in a dendro-axonal fashion, e.g. there are also axo-axonal synapses modulating anterograde action potentials or the depolarization of the presynaptic site. This implies that certain NT receptors also can be transported to the axon such as some metabotropic glutamate receptor subunits [11]. Moreover, myelinated axons show different protein composition under the myelinated sheath than at the nodes of Ranvier [12]. Additionally, not all postsynaptic densities of one cell contain the same receptors; the receptor distribution is specific for the NT released from the presynaptic site [3]. Finally, even postsynaptic densities with the same receptor type may differ in their morphology and protein composition due to different stimuli from the presynaptic site.

The multiple subdomains on the plasma membrane highlight the elaborate sorting achieved by neurons. To understand how neurons achieve this compartmentalization one useful approach is to conceptualize neuronal architecture as two domains: axonal and dendritic. This allows one to make a comparison between neurons and epithelial cells in which plasma membrane is separated into apical and basolateral and the sorting mechanisms are much better understood. Although such studies have their limitations, as will be discussed later, they have helped to unravel some of the sorting signals operating in neurons.

1.1. The neuronal/epithelial sorting hypothesis

Early studies on membrane sorting in neuronal cells relied on the use of different RNA viruses: Semliki Forest virus (SFV), vesicular stomatitis virus (VSV), and fowl plague virus (FPV). The rationale behind this strategy is simple. These viruses allow to distinguish between the apical and basolateral domain in polarized epithelial cells and could be used to study axonal and dendritic sorting. Infection of epithelial cells with the FPV leads to apical delivery of hemagglutinin (HA) whereas infection with SFV or VSV leads to basolateral delivery of the E2 and G viral proteins, respectively [13–15]. Upon infection of polarized hippocampal neurons with the same viruses HA is largely delivered to the axon while the E2 and G proteins are delivered to the dendritic surface [16–18]. These results show (1) a similarity between the axonal and the apical membrane domain as well as between the dendritic and the basolateral membrane domain and (2) the existence of a direct routing pathway from the *trans*-Golgi network (TGN) in the cell body to the axonal and to the dendritic plasma membrane. It suggests that the mechanisms controlling sorting in epithelial and neuronal cells may be similar.

An important criterion for this hypothesis is the distribution of endogenously expressed proteins. GPI anchored proteins shown to be apically transported

in Madin-Darby canine kidney (MDCK) cells, a polarized epithelial cell line, are axonally localized in hippocampal neurons in culture [19,20]. The γ-aminobutyric acid (GABA) transporter is axonal in neurons and apical if expressed in polarized epithelial cells [21]. Somatodendritic-basolateral expression is found for the transferrin receptor [22–25], for the LDL receptors (De Strooper and Dotti, manuscript in preparation), for the α-subunit of the $GABA_A$ receptor [31,32], and for the human epithelial polymeric immunoglobulin receptor (pIgR) [26,27]. In addition, a transcytotic pathway existing for the pIgR in MDCK cells [28–30] also exists in neurons in a similar fashion: upon viral expression pIgR first appears in the dendritic plasma membrane and then redistributes to the axonal domain [27].

Nevertheless, there are also discrepancies to the hypothesis of analogue membrane domains in neurons and epithelial cells. The β-subunit of the $GABA_A$ receptor is somatodendritically distributed in hippocampal neurons but it is apically transported when expressed in MDCK cells. The β-subunit even redirects the α-subunit to the apical side upon cotransfection in MDCK cells. Another protein contradicting the hypothesis is the amyloid precursor protein (APP) which is basolateral in epithelial cells and axonal in neurons [33–38,132]. The hypothesis is also contradicted by the immunofluorescence demonstration that a (Na+K)-ATPase is present in both axon and dendrites of hippocampal neurons but only in the basolateral surface of epithelial cells [39]. However, this is caused by specific retention of this protein mediated by the cytoskeletal linker protein ankyrin, rather than by a difference in sorting [40]. In epithelial cells, the (Na+K)-ATPase is delivered to the apical and the basolateral side but retention is only on the basolateral side of the protein [41]. The importance of retention is further supported by results showing that retinal pigment epithelium cells have the ankyrin-fodrin cytoskeletal linker proteins attached to the apical side where also the (Na+K)-ATPase localizes [42].

In conclusion, some proteins contradict the theory of sorting similarities between epithelial and neuronal cells. This is not surprising since different epithelial cells of different origins sort the same proteins in a different manner. Indeed, the LDL receptor is apical in the epithelial cells of the kidney tubule and basolateral in the epithelial cells of the intestine and liver and the same GPI-anchored proteins are apical in kidney epithelial cells and basolateral in thyroid epithelial cells [43,130]. Given that cells derived from the same origin do not use the same sorting mechanisms, it is extreme to think that this will be the case for cells so diverse as neurons and epithelial cells. When thinking of how the plasma membrane of any given cell is made, one must accept that sorting mechanisms are flexible and complex in order to generate cell specificity. Hence, the main goal now should be to understand the mechanisms underlying neuronal sorting.

1.2. Axonal and dendritic trafficking in fully polarized neurons: the sorting signals

Studies on the sorting signals of membrane proteins in neuronal cells profited from the use of viral vectors as a tool for protein expression. The first axonal sorting signals were identified in APP [38]. This protein is found in axons of central nervous system neurons both in vitro and in situ. Expression of APP in cultured hippocampal neurons with the SFV expression vector results in its rapid axonal transport [36] similar to the endogenous form [45]. Interestingly, expression of this neuronal protein in MDCK epithelial cells results in its basolateral delivery [33,34]. In MDCK cells the basolateral sorting information is contained in a short stretch of amino acids in the cytoplasmic tail, but the ectodomain controls basolateral secretion [33–35,132]. In neurons, deletion experiments have shown that the axonal sorting information is present in the transmembrane-ectodomain (see Fig. 2) and deletion of the cytoplasmic tail does not affect axonal targeting [38], showing that epithelial and neuronal cells process this protein in a cell-specific form. Since this is a neuronal protein one possible explanation is that epithelial cells do not have the sorting machinery capable of recognizing the axonal sorting information of this protein as apical.

The importance of the transmembrane-ectodomain as the site for axonal sorting information is not general. Recent work demonstrates that a different axonal membrane protein, synaptobrevin, contains sorting information in the cytoplasmic domain [46] (see Fig. 2). In this work it was shown that a chimera

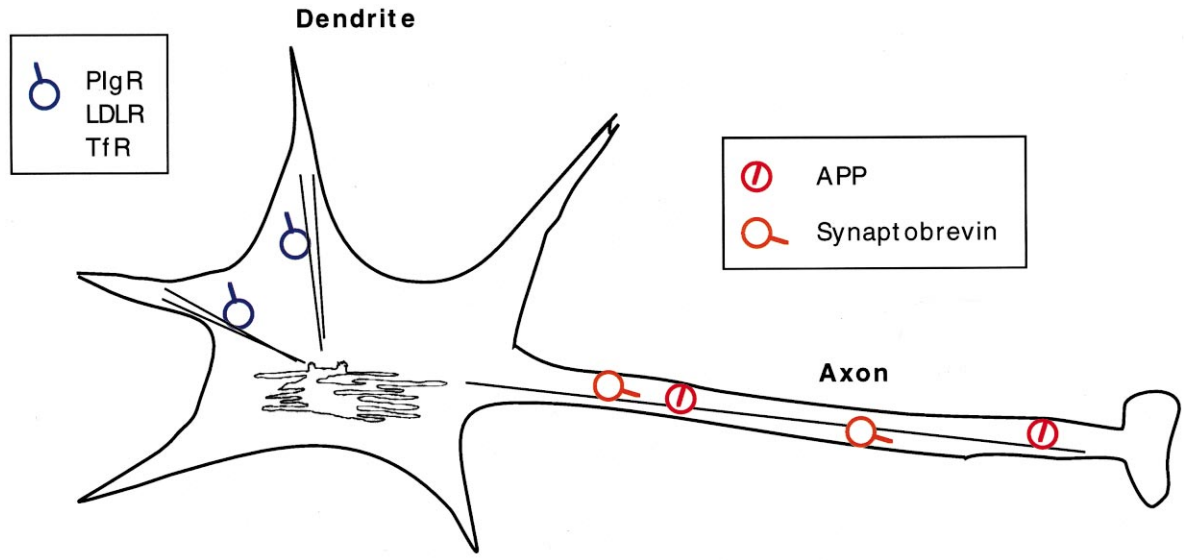

Fig. 2. Sorting signals of axonal and dendritic membrane proteins. Axonal and dendritic proteins exit the TGN in different transport carriers (dendritic: blue; axonal: red, orange) which are moving on microtubules (black straight lines). Dendritic proteins such as the polymeric immunoglobulin receptor (PIgR), the transferrin receptor (TfR) and the low density lipoprotein receptor (LDLR) have their sorting signals in the cytoplasmic tail (represented as blue line outside of the dendritic transport structure). The axonal protein APP comprises its sorting signal in the ectodomain/transmembrane domain (red line enclosed by an axonal transport structure). This, however, does not seem to be the case for synaptobrevin, another axonal protein (orange) which contains a cytoplasmic axonal sorting motif.

protein containing the cytoplasmic domain of synaptobrevin added to the complete transferrin receptor, which alone is sorted to the dendrites, is capable of directing the transferrin chimera protein to the axonal territory [46]. The different sorting mechanisms of these two axonal proteins, APP and synaptobrevin, highlight the complexity of the neuronal sorting machinery and suggest that several different sorting mechanisms must operate for the correct delivery of membrane constituents to the axonal surface. Thus, one possibility is that synaptobrevin and APP use different sorting mechanisms to deliver the proteins to different subdomains.

The dendritic sorting signals have been identified in pIgR [26], transferrin receptor (TfR) [23] and low density lipoprotein receptor (LDLR) (De Strooper and Dotti) (see Fig. 2). All these proteins are delivered to the basolateral membrane in MDCK cells. A preferential dendritic delivery of the newly synthesized protein pIgR is observed upon infection of hippocampal neurons with recombinant SFV encoding pIgR [26]. In epithelial cells expressing pIgR the basolateral sorting information is contained in a short segment of the cytoplasmic tail (CT) that is 14 amino acids long (position 655–668: RHRRNVDRVSICSY) [44]. Its deletion leads to apical delivery. When the same mutant pIgR is expressed in fully polarized neurons the newly synthesized protein appears on the axonal surface [27]. However, at later times post infection the mutant protein is also found on the dendritic surface. Dendritic transport must be because of missorting and not because of transcytosis since deletion of the CT blocks endocytosis. These results have two important implications. On the one hand they highlight the importance of the amino acid motif in the CT for dendritic delivery (see Fig. 2). On the other hand, the late appearance of mutant pIgR suggests that the axonal pathway is easily saturable because the CT minus pIgR appears in the dendrites at late expression times. This is a common result with overexpressed axonal proteins in cultured hippocampal neurons and has been also observed for the influenza virus hemagglutinin (Ledesma and Dotti, unpublished results), and APP [36] expressed in these cells. Hence, to analyze whether or not a given protein is sorted to the axonal surface, it appears necessary to use fully polarized neurons in which endogenous

membrane as well as cytoskeletal proteins are correctly positioned, and a transfection method that allows a close temporal follow-up between protein expression and protein distribution.

As opposed to the axonal sorting machinery, dendritic missorting to the axon is rarely seen. It is possible that the larger dendritic surface area accounts [121] for this difference in the sorting fidelity between the axonal and dendritic pathways. However, until we know more about the molecular machinery underlying membrane sorting it will not be possible to solve this issue.

1.3. Proteins enriched in synaptic specializations: sorting vs. retention

Sorting of membrane proteins from the TGN into different vesicles is one way to achieve asymmetric localization of proteins. However, proteins are also heterogeneously distributed by specific retention, i.e. a mechanism by which membrane proteins are unselectively transported to different plasma membrane territories and only become retained upon specific binding to other proteins, thereby avoiding inclusion in transporting endosomes. One example for retention is the (Na+K)-ATPase. It is transported to both the apical and basolateral surfaces in MDCK cells but becomes only stabilized on the basolateral surface through interaction with cytoskeletal linker proteins [40,41]. In neurons similar mechanisms could determine the formation of microdomains. In this section the selective enrichment of proteins in the postsynaptic density is discussed.

The postsynaptic density (PSD) is specialized in synaptic transmission. It contains a large number of NT receptors, ion channels and pumps [2,47]. The clustering of NT receptors was initially analyzed from preparations of neuromuscular junctions, the structure at which the axonal terminal of motor neurons connects skeletal muscle cells. This structure has the advantage that synaptic components are easily purified and that NT receptors are highly enriched (10^4 nicotinic acetylcholine receptor (nAchR) molecules per square micrometer are found on the PSD) while non-synaptic areas are practically devoid of AchR molecules [48–50]. Clustering of NT receptors is mainly induced by extracellular clues but can also occur in the absence of neuritic innervation. Hence, much research focused onto these two aspects: (a) which and how extracellular signals trigger and maintain clustering and (b) which intracellular components are necessary for the same event.

To be important for receptor clustering an intracellularly acting candidate should fulfill several criteria: (1) its coexpression should lead to the clustering of the membrane protein; (2) inhibition of the expression of the associated protein should prevent clustering; (3) the associated protein should bind in vitro to the membrane protein; (4) it should colocalize in vivo; (5) it should be present at the earliest time points of receptor clustering; (6) it should also have a role in linking the receptor cluster to the cytoskeleton, either by direct binding or via other associated proteins, in order to arbour the cluster at the correct position. A good candidate for clustering nAchR molecules is the cytosolic protein rapsyn, formerly called 43 kDa protein, which copurifies with AchR protein [51–53]. Rapsyn coexpressed with AchR in the quail fibroblast cell line QT-6 and in *Xenopus* oocytes clusters AchR on the surface [54,55]. AchR-rich membrane depleted of rapsyn by alkaline treatment results in disordering of the AchR clusters [56–59] and rapsyn deficient mice do not show clustering of receptor in muscle fibers [60]. Crosslinking experiments show specific interaction of rapsyn with the β-subunit of AchR [61] although any AchR subunit individually coexpressed with rapsyn has the potential to cluster [62]. Rapsyn interacts through β-dystroglycan and utrophin with the actin cytoskeleton [63,64]. Rapsyn colocalizes with the earliest nAchR clusters in embryonic mouse muscle [65] as well as in *Xenopus* muscle cell culture [66,67]. However, in the developing electric organ in *Torpedo* muscle where the first nAchR form before innervation takes place, rapsyn is not colocalized with the nAchR clusters [68,69]. This last result suggests that rapsyn is not the only factor that can cluster this receptor. Indeed, conditioned media from a neuroblastoma-glioma cell line and extracellular matrix isolated from the electric organ of *Torpedo* trigger AchR clustering in fibroblasts stably transfected with nAchR but lacking rapsyn [70].

The importance of extracellular clues in cluster formation is evident from the observation that regenerating myotubes form clusters only at the same positions where they were present before damage oc-

curred when an intact basal lamina is present [71–73]. The basal lamina at the synaptic specialization is biochemically distinct and its characterization led to the discovery of the basal lamina protein agrin. Agrin causes clustering of nAchR [74–76], but the mechanism of action is not yet understood. Agrin induced clustering appears to act in a catalytic fashion rather than by direct interaction between agrin and nAchR. Whereas rapsyn and AchR occur roughly in a 1:1 stoichiometry in the PSD, one agrin molecule can cause 100 AchR molecules to cluster [77]. Beside its effect on clustering AchR, agrin also induces tyrosine phosphorylation and inhibition of phosphorylation blocks agrin-mediated clustering [78–82]. In addition, agrin binds directly to α-dystroglycan. This extrinsic peripheral protein is covalently attached to β-dystroglycan which in turn binds to the intracellular cytoskeleton through dystrophin and the dystrophin-related protein utrophin [83–87]. Hence, it is probable that the agrin induced clustering may be linked to the rapsyn clustering via β-dystroglycan and utrophin.

The success in identifying the components that cluster nAchR at the postsynaptic densities in the neuromuscular junction also triggered the search for proteins with receptor clustering ability in the axo-dendritic synapses. The first protein discovered was gephyrin, which seems to be responsible for glycine receptor (GlyR) cluster formation. Gephyrin copurifies with the GlyR as a peripheral membrane protein [88,89]. GlyR clusters with gephyrin upon coexpression in human embryonic kidney 293 cells [90]. Interestingly, GlyR clusters not only at the plasma membrane with gephyrin but also in intracellular gephyrin aggregates. Moreover, the heterologously expressed GlyR subunits as well as GlyR β-subunit alone are re-routed to intracellular gephyrin aggregates. This is not the case for the GlyR α-subunit suggesting that the β-subunit contains the gephyrin binding motif. Clustering of GlyR is inhibited in the absence of gephyrin mRNA and protein in cultured spinal neurons [91]. Gephyrin binds to an 18 amino acid containing motif located in a cytoplasmic loop of the GlyR β-subunit [92]. Insertion of the gephyrin binding domain into the homologous cytoplasmic loop of the $GABA_A$ receptor enables it to bind to gephyrin and to cluster when coexpressed with gephyrin in 293 cells while most of the wild type $GABA_A$ receptor neither binds to gephyrin in vitro nor clusters upon coexpression with gephyrin in this kidney cell line [92]. Gephyrin colocalizes in vivo with GlyR in immunofluorescence and electron microscopy [2,47,91,93,94] and there is some indication that gephyrin aggregates are present at the plasma membrane before GlyR [91,94,95]. Gephyrin can directly bind to microtubules [96,97] and upon pharmacological manipulation of the cytoskeleton the distribution of the gephyrin aggregates changes [98]. For other NT receptor proteins less is known. The *N*-methyl-D-aspartate (NMDA) receptors, a subclass of the ionotropic glutamate receptors, comprise two families of homologous subunits, the principal NR1 subunit and different modulatory NR2A-D subunits. The NR1 subunit of the NMDA receptor clusters when expressed in fibroblast [99] and is regulated on two levels, transcriptionally and posttranslationally. NR1 expression alone is sufficient for clustering in QT-6 cells, a result different from the nAchR and the GlyR. NR1 clustering occurs when the first carboxy terminal exon cassette is present. Upon insertion of the same cassette into GluR1, a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazol propionate (AMPA) receptors, the other ionotropic glutamate receptor subclass, the behavior of GluR1 changes from a uniform to a clustered distribution. Hence, the carboxy terminal exon cassette seems to be necessary and sufficient to regulate clustering. Moreover, the cassette contains specific protein kinase C phosphorylation sites. Treatment of the cells with phorbol ester inducing phosphorylation of these sites causes the clusters to be reversibly dispersed. Mutation of two of the serines to alanines in this cassette inhibits dispersion of the clustering [99].

Some NR1 splice variants (NR1-3, NR1-4 [133,134]) of the NMDA receptor subunits as well as NR2 subunits interact with the postsynaptic density protein PSD-95 as shown by interaction in the yeast two-hybrid system as well as by coimmunoprecipitation [100]. The interaction is mediated through the second PDZ domain in PSD-95 and a carboxy terminal motif of the NMDA receptor subunits referred to as t/SXV motif [100]. The t/SXV motif is also found in K^+ channels and consist of a serine or threonine followed one amino acid later by a valine. The PDZ protein family, named after PSD-95, the

Drosophila tumor suppressor gene *lethal (1) disc large* (Dlg-A) and the human tight junction protein zonula occludens-1 (ZO-1), seems to be involved in binding or clustering of other proteins at the membrane at places of cell-cell contact. Dlg-A, for example, is localized in the presynaptic and postsynaptic membrane of a subgroup of synaptic boutons at the neuromuscular junction of *Drosophila* [101]. Mutation of this gene alters the subsynaptic reticulum, a postsynaptic specialization.

The members of the rapidly growing PDZ family have three shared domains: one domain homologous to a yeast guanylate cyclase [102], a src homology 3 (SH3) domain, and at least one PDZ domain, also referred to as GLGF motif. Two members of the PDZ family, human homologue of dlg and p55, can bind to the cytoskeleton through protein 4.1 [103,104], suggesting that this family of proteins act as a linker between t/SXV motif carrying receptors and the cytoskeleton.

For correct positioning on the plasma membrane both sorting and retention may complement each other. Thus, it is likely that proteins are delivered to the correct destination using the described sorting information and then, after fusion, retention signals may specifically anchor the membrane protein to the cytoskeleton and other proteins. There has been some evidence that insertion of nAchR into the plasma membrane is not a random process, but preferentially takes place at clusters in cultured myotubes [106,107]. Hetero-oligomeric GlyR as well as its β-subunit alone are clustered in intracellular gephyrin-rich aggregates upon coexpression with gephyrin in the 293 cell line [90]. Membrane receptors often are synthesized before synapse formation [94] and in the case for GluR1 and GlyR they exist in intradendritic stores before cluster formation takes place [91,108,109]. However, it has not been investigated whether insertion from these pools into the plasma membrane takes place at specific sites or randomly. There may be the need of direct routing of proteins to achieve specificity. PSD-95, for example, can interact with both the glutamate receptor subunit NR2 and the Shaker-type K^+ channel Kv1.4 [100,105], but NR2 is found on the dendrites while the Shaker-type K^+ channel localizes on subdomains of the axon [100,135]. Possibly, these proteins may be transported on different carriers after leaving the TGN as demonstrated for non-synaptic proteins (see above) and PDZ-like proteins would then act during retention. But this remains to be investigated.

1.4. Establishment of neuronal polarity

The mechanisms discussed so far concern mature neurons but how does a neuron become polarized? When is the sorting and clustering machinery mature enough to specifically deliver and anchor cargo to the axon and the dendrites? Most studies addressing this question are performed in cell culture systems, mainly in hippocampal neurons derived from rat embryos which readily differentiate axons and dendrites [110,111]. These neurons develop in well defined stages and in a stereotypic fashion [112]. Shortly after plating the cells attach and form lamellipodia around the cell body (stage 1). After 1 day in culture the lamellipodia condenses and forms several neurites that become 10–25 μm long. These neurites are similar to each other but are different from the cell body in many respects, e.g. microtubules are bundled in the neurites but form a netlike structure in the cell body; the Golgi complex is located in the cell body but is absent in the neurites [116]. (Note that the Golgi apparatus is also localized to the cell body in mature neurons and does not extend to the distal neurites [136].) Within the next 24 h one of the neurites starts to grow very rapidly whereas the other neurites stay quiescent (stage 3). The growing neurite becomes the axon of later stages. Stage 3 is therefore the first manifestation of polarization. The other neurites start to grow slower after 4–6 days in culture (stage 4) and will later form the dendrites. After 10 days in culture the axons and dendrites form synapses (stage 5). Although hippocampal neurons have an intrinsic program to form only one axon, there appears to be a high degree of flexibility before the final decision is made. All neurites have the potential to become an axon as has been shown when axons of stage 3 cells were lesioned [113,114]. In less flexible neurons, such as the Ti1 unipolar neuron in the grass hopper nervous system, the microtubule organizing center (MTOC) and the Golgi apparatus are positioned at the site of the future axon before axonal outgrowth takes place [115]. A similar analysis in the multipolar hippocampal neurons shows that in this system such a correlation does not exist [116]. Hence,

other mechanisms may determine which of the multiple processes becomes the axon. Since the axonal membrane proteins synapsin 1, synaptophysin, synaptotagmin and GAP-43 are enriched in the growing axon of stage 3 cells [117–120] one may envision that the appearance of membrane sorting is the determinant of axon formation [118,121]. An alternative view is that the initial polarization events of a neuron are caused by a general cytoplasmic flow including axonal as well as dendritic transport structures into one neurite that then triggers axonal outgrowth. This situation is similar to migrating non-neuronal cells in which membrane flow in the form of exocytic vesicles and microtubule stabilization is polarized towards the leading edge of the cell [122–125]. Indeed, two publications suggest that this is the case in neurons [126,127]. Recent experiments in our group show that a membrane flow towards the growing axon exists [127]. This flow contains TGN-derived vesicles. Furthermore, beside the enrichment of axonal membrane proteins in the growing axon the AMPA receptor subunit GluR1 is also found concentrated in the growing axon. This receptor subunit is typically found in dendrites in adult neurons [3,128]. The vectorial cytoplasmic flow also contains components that are not directly involved in membrane transport and insertion such as mitochondria, peroxisomes and the cytosolic protein iron regulatory protein. These results suggest that neuronal maturation is a two-step phenomenon in which maturation is first morphological (independent of molecular sorting) and then functional (requiring sorting and anchoring). Consistent with this, Ledesma et al. (submitted) showed that certain axonal membrane proteins which normally interact with sphingomyelin and cholesterol in adult neurons do not show this interaction in young neurons.

Based on this, establishment of functional polarity will be dependent on the maturation of many components. The sorting machinery in the Golgi apparatus has to mature in such a way that axonal and dendritic cargo can be sorted into distinct vesicles. Maturation of the transport machinery must occur to send the segregated cargo to either the axon or the dendrites. This implies the expression of microtubule associated and mechanochemical proteins to support traffic along axonal and dendritic microtubules. This seems to occur before the maturation of the Golgi sorting pathway as different MAPs, motor proteins, as well as the formation of microtubules of mixed polarity appear polarized very early in development, coincident with dendritic growth (stage 4) [129]. Thus, the cytoskeleton appears to be mature before stage 5, when the sorting and anchoring machinery is properly functioning. Glutamate receptors are sorted to the dendrites before synaptogenesis but only cluster after synapse formation [3]. Besides the maturation of the anchoring machinery for synaptic proteins one would envision that the fusion machinery specific for axonal and dendritic cargo probably using specific v-SNAREs/t-SNAREs must also mature. These aspects still remain to be investigated.

2. Conclusion

Neurons reach functional maturation by a series of sequential events. First, there is a morphological differentiation. The axons are formed using intracellular mechanisms common to migrating non-neuronal cells. Later, molecular segregation of axonal and dendritic protein occurs using sorting motifs placed in the cytoplasmic tail or in the transmembrane-ectodomain. There are probably more routing pathways than one axonal and one dendritic reflected by the different axonal sorting motif positions. This may be one origin of the formation of subdomains within neurons. Subdomain formation also depends on retention of specific membrane protein on the plasma membrane linked at the correct positioning to the cytoskeleton. The interplay between sorting and routing of proteins and their specific retention remains to be investigated.

The hypothesis of neuronal and epithelial membrane traffic similarity reaches its natural end. It showed substantial success in unraveling sorting pathway in neurons by applying tools and models derived from the work with epithelial cells onto neurons. However, we reached a knowledge about the sorting signals, retention signals and the development of sorting pathways which stresses the specific properties of neurons themselves. We think that future research on neuronal polarity should focus now on the concrete sorting mechanisms in neurons.

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