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The Polarized Sorting of Membrane Proteins Expressed in Cultured Hippocampal Neurons Using Viral Vectors

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Summary

One model of neuronal polarity (Dotti and Simons, 1990) proposes that neurons and polarized epithelia use similar mechanisms to sort membrane proteins. To explore this hypothesis, we used viral vectors to express proteins in cultured neurons and assessed their distribution using quantitative immunofluorescence microscopy. Basolateral epithelial proteins were polarized to dendrites; more significantly, mutations of sequences required for their basolateral targeting in epithelia also disrupted dendritic targeting. Unexpectedly, apical proteins were not polarized to axons but were expressed at roughly equal amounts in dendrites and axons. These data provide strong evidence that targeting of basolateral and dendritic proteins depends on common mechanisms. In contrast, the sorting of proteins to the axon requires signals that are not present in apical proteins.

Introduction

Nearly every aspect of neuronal function depends on the polarized targeting of membrane proteins to dendrites and axons. The accurate localization of ion channels, neurotransmitter receptors, and the proteins that mediate neurotransmitter release plays an integral role in neuronal information processing. The targeting of cell adhesion molecules and growth factor receptors is likely to play an equally critical role in regulating the outgrowth of neurites and the formation of synaptic connections during neural development.

The hypothesis that neurons and epithelial cells use common cellular mechanisms to generate a polarized distribution of membrane proteins has given important impetus to the study of protein targeting in neurons (Dotti and Simons, 1990). The basis for this model derives from a study of the targeting of viral proteins in neurons infected with vesicular stomatitis virus (VSV) or influenza virus, following the approach pioneered by Rodriguez-Boulan and Sabatini (1978) that helped to define the cellular pathways underlying the polarization of membrane proteins in the Madin-Darby canine kidney (MDCK) epithelial cell line. Dotti and Simons (1990) concluded that VSV G protein, which is targeted to the basolateral domain of MDCK cells, was polarized to the somatodendritic domain in cultured hippocampal neurons; influenza hemagglutinin (HA), which is targeted to the apical domain in MDCK cells, was polarized to the axon. Subsequent work comparing the distribution of proteins in the two cell types tends to support the parallel between basolateral and dendritic sorting and between apical and axonal sorting, although several exceptions have been noted (see Discussion).

In the current study, we used replication-defective herpesvirus (HSV) or adenovirus (AdV) to express the following proteins in polarized neurons: three basolateral marker proteins, sorting signal mutants of these proteins that disrupt their basolateral targeting, three apical markers, and the chick homolog of an endogenous axonal protein. Only a small percentage of the neurons was infected in our experiments, allowing the distribution of the expressed proteins to be accurately analyzed in individual neurons using quantitative immunofluorescence microscopy. As predicted, we observed that basolateral proteins were targeted to dendrites; moreover, mutation of the same sequences responsible for basolateral targeting in epithelia also disrupted dendritic targeting. In contrast, apical proteins were not targeted to axons; rather, they were expressed in both dendrites and axons. These results demonstrate that the sorting of proteins to the axon requires signals that are not present in apical proteins.

Results

Endogenous Proteins Are Appropriately Polarized When Expressed Using Viral Vectors

Our strategy to elucidate mechanisms underlying protein targeting in hippocampal neurons was based on expressing a variety of wild-type and mutant protein constructs using defective viral vectors. In preliminary experiments (data not shown), we examined the time course of appearance of proteins on the cell surface following infection with a defective HSV expressing the lymphocyte protein CD8α and an AdV expressing the low density lipoprotein receptor (LDLR). Cell surface expression from either virus was first detectable in a few cells starting at 8 hr after infection. The percentage of expressing cells increased markedly between 8 and 12 hr, with a further modest increase between 12 and 18 hr after infection. The level of expression of cell surface protein in infected cells increased for up to 36 hr after infection, the longest time point examined. This time course is consistent with previous reports of expression from defective HSV in hippocampal cultures (Craig et al., 1995).

To determine if neurons appropriately segregated proteins that were overexpressed with viral vectors, we expressed species homologs of two endogenous neuronal proteins, the transferrin receptor (TfR), a somatodendritic protein (Cameron et al., 1991; Mundigl et al., 1993), and the cell adhesion molecule L1, an axonal protein (van den Pol and Kim, 1993). Virus was added

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to 13-day-old cultures, and the distribution of the expressed proteins was assayed using quantitative immunofluorescence microscopy after 18 and 36 hr. Human TfR was expressed via defective HSV and localized with a human-specific antibody (Omary and Trowbridge, 1981). Like endogenous TfR, the expressed human protein was polarized to the somatodendritic domain (Figure 1). The cell's dendrites, defined by MAP2 labeling, were brightly stained, whereas staining of the axons of infected cells was often so low that they could not be distinguished from the MAP2-negative processes that arose from nearby uninfected cells. On average, 98% of TfR staining was associated with dendrites at both 18 and 36 hr after infection. NgCAM, the chick homolog of L1, was expressed with defective AdV and visualized by immunofluorescence using a species-specific antibody (Lemmon and McLoon, 1986). NgCAM was polarized to the axon (Figure 1). Labeling was evident throughout the entire extent of the cell's axonal arbor, being especially concentrated in the distal portion of the axon. The level of dendritic staining was near that of uninfected cells. On average, 96% of the NgCAM staining 18 hr after infection was axonal. These data show that overexpression of proteins by viral vectors does not alter their distribution relative to their endogenous counterparts.

Distribution of Basolateral Proteins and Their Sorting Signal Mutants

We examined the localization of three representative basolateral proteins—polyimmunoglobulin receptor (pIgR), LDLR, and TfR—as well as mutations of each protein that are known to disrupt their sorting in epithelial cells. The pIgR was expressed with defective HSV in 14-day-old cultures. In polarized epithelia, pIgR is first targeted to the basolateral surface and then transcytosed to the apical surface (Mostov and Deitcher, 1986). At short times after infection, cell surface expression of pIgR was polarized to the dendrites in hippocampal neurons (Figure 2). At 12 hr after infection, staining was observed throughout the dendritic arbor, whereas axonal labeling was restricted to the most proximal segment. At later times after infection, the extent of axonal labeling increased and the distribution of label extended more distally along the axon. Quantitative analysis revealed that the mean percentage of total staining associated with the dendrites decreased with time of expression from 97% after 12 hr of infection to 75% after 36 hr (Table 1). These data suggest that pIgR is targeted initially to dendrites in a highly polarized fashion; the subsequent appearance of axonal labeling may represent transcytosis, although its time course and distribution are also consistent with diffusion within the plane of the plasma membrane.

We then examined the distribution of a mutant of pIgR (pIgR 655–668) that lacks the basolateral sorting signal (Casanova et al., 1991). In all cells expressing pIgR 655–668, staining was observed on the surface of both dendrites and axons at all times (Figure 2). In marked contrast to wild-type pIgR, only about 30% of the pIgR 655–668 was associated with dendrites, a value that did not change significantly with time of expression (Table 1). The distribution of pIgR 655–668 within the axonal arbor was also distinctly different from that of wild-type pIgR. It appeared to extend throughout the entire axonal arbor, with particularly prominent labeling of axonal growth cones (a pattern similar to that previously observed for other proteins expressed on the axonal surface; Craig et al., 1995; Vogt et al., 1996).

The basolateral marker LDLR was expressed with defective AdV in 14-day-old cultures. In polarized epithelia, LDLR is targeted to the basolateral surface (Hunziker et al., 1991). In hippocampal neurons, cell surface expression of LDLR was highly polarized to the dendrites (Figure 3). Staining of non-MAP2 processes was so low that the unambiguous identification of the cell's axon beyond 200 μm from the cell body was difficult. The extent of polarization did not significantly change with time of expression (Table 1).

LDLR contains two distinct and redundant basolateral sorting signals in its cytoplasmic tail. To determine the role of these signals in the dendritic targeting of LDLR, we expressed mutants that independently disrupt each of the two signals and a mutant in which both signals are disrupted. The first (LDLRY18 → A) contains a point mutation of tyrosine that abolishes basolateral targeting information in the membrane-proximal sorting determinant. The second was a truncation that eliminates the membrane-distal sorting determinant (LDLRCT33). In epithelia, both of these mutants are still targeted to the basolateral surface, since one of the two signals remains intact. The third construct we examined contained point mutations of essential tyrosine residues in both sorting signals (LDLRY18,35,37 → AAA). These mutations eliminate basolateral targeting in MDCK cells (Matter et al., 1994). Mutations that affected only one of the two sorting signals had no significant effect on the dendritic targeting of LDLR. In contrast, when both sorting signals were disrupted, selective targeting to dendrites was abolished (Figure 3). Like mutant pIgR, the axonal staining of this LDLR mutant extended throughout the arbor and the most intense staining was observed in the growth cones and distal tips of the axon.

Finally, we examined the distribution of a mutant of the TfR that lacks the cytoplasmic tail (TfR 3–59). When expressed in MDCK cells, this mutant is found on both basolateral and apical surfaces (Kundu and Nayak, 1994; Odorizzi et al., 1996). In hippocampal neurons, cell surface expression of TfR 3–59 was observed on both dendrites and axons (data not shown). Staining extended throughout the dendritic arbor. Axonal staining was evident only proximally; expression on axonal growth cones and distal axons was not observed. The dendritic polarization of TfR 3–59 was significantly less than that of wild-type TfR (66% versus 98%) at both 18 and 36 hr after infection (Table 1).

Distribution of Apical Proteins Expressed in Hippocampal Neurons

While the results just described are consistent with the parallel between basolateral and dendritic sorting, one aspect of these results was unexpected. When basolateral sorting signals are deleted from pIgR and LDLR, these proteins are targeted to the apical domain of

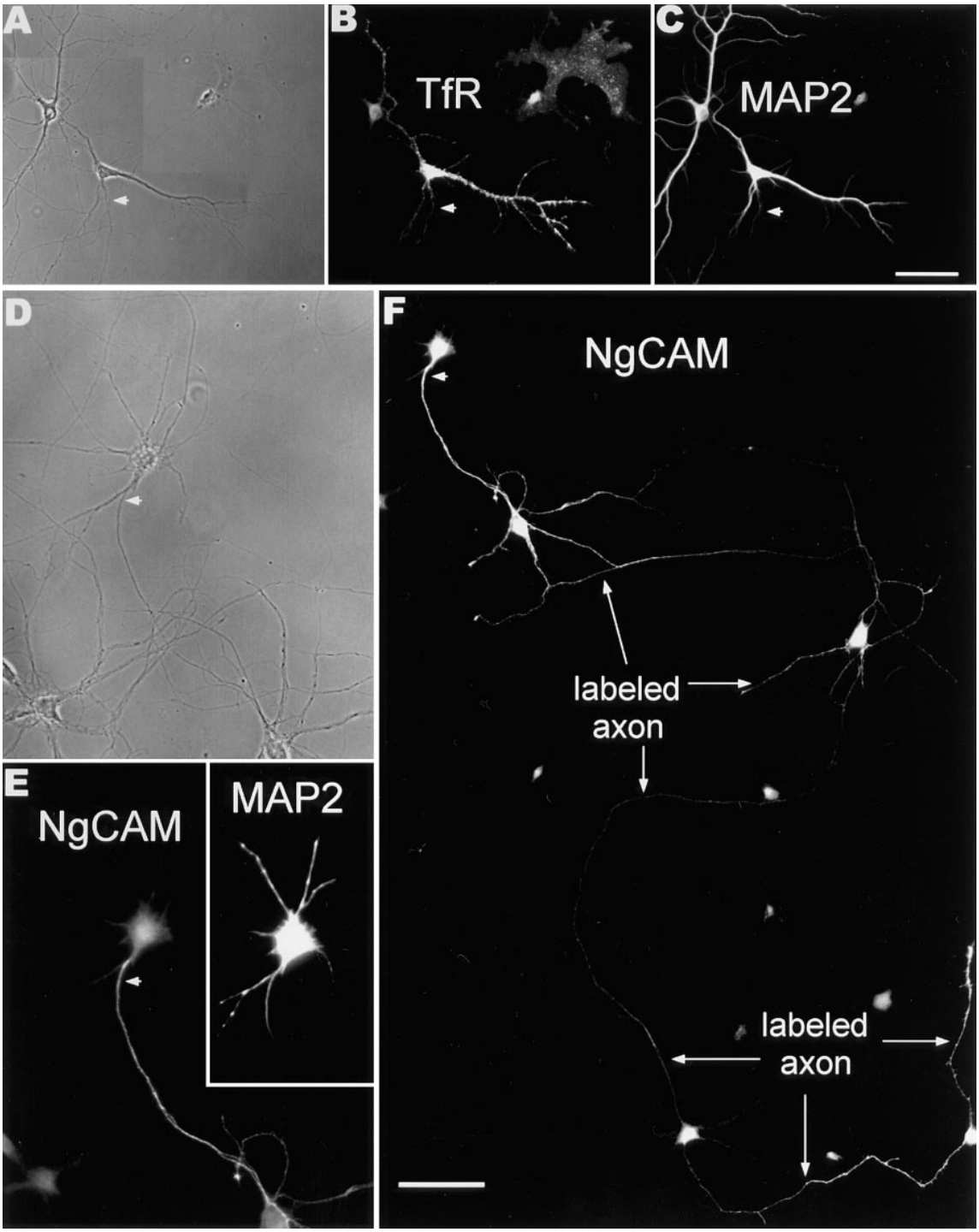

Figure 1. Homologs of Endogenous Axonal and Somatodendritic Proteins Are Appropriately Polarized When Expressed in Hippocampal Neurons with Viral Vectors

(A–C) The distribution of human TfR following expression with replication-defective HSV. (A) shows a phase contrast micrograph of a representative field from a 14-day-old culture. (B) and (C) illustrate the distribution of human TfR and endogenous MAP2, respectively. One of the two neurons in this field, along with one glial cell, express human TfR. In the neuron, labeling was restricted to the cell body and dendrites (identified by MAP2 labeling). Unambiguous identification of the axon of the TfR-expressing cell was not possible; the most likely candidate is indicated by the arrowhead. Scale bar, 100 μm.

(D–F) The distribution of NgCAM, the chick homolog of L1, following expression with a replication-defective AdV. (D) is a phase-contrast micrograph from a 14-day-old culture, and (E) is a fluorescence micrograph of the same field illustrating the distribution of NgCAM. This field contains the somata of four neurons, one of which expresses NgCAM. Cell surface NgCAM was highly polarized to the axon, with only light labeling of proximal dendrites (identified by MAP2 labeling, inset). (F) shows a lower magnification view of the same labeled cell to illustrate that NgCAM staining extends throughout the cell's entire axonal arbor; axonal staining appeared most intense in the distal portions of the axon. Nonspecific staining of the cell bodies of uninfected cells is also evident in these micrographs. This reflects the much greater thickness of somata compared with either dendrites or axons. Scale bar, 100 μm.

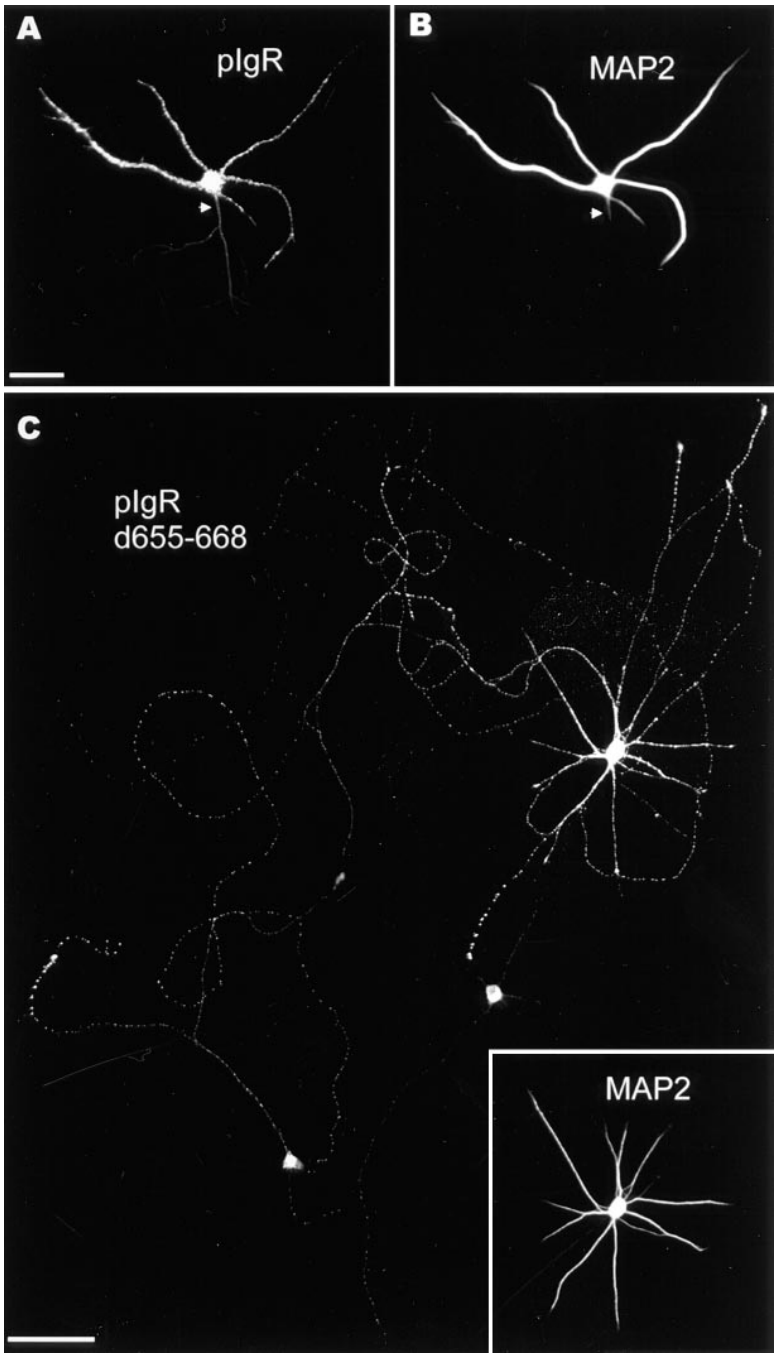

Figure 2. The Distribution of the Basolateral Protein pIgR and the Basolateral Sorting Signal Mutant pIgR 655–668 following Their Expression in Hippocampal Neurons

Cell surface labeling of wild-type pIgR (A) was polarized to dendrites, which were identified by MAP2 labeling (B). Staining was also present in the proximal axon, though at lower intensity than in the dendrites. In contrast, the basolateral sorting signal mutant of pIgR did not exhibit a polarized distribution. In addition to dendritic staining, the mutant pIgR (C) extended throughout the axonal arbor and was most intense in distal portions of the axon. The cells shown were examined 18 hrs after viral infection. Inset in (C), MAP2 staining. Scale bar, 50 μm.

MDCK cells. In contrast, these mutants were not selectively targeted to the axons of hippocampal neurons. To assess the targeting of apical proteins more systematically, we expressed three other representative apical proteins in 14-day-old hippocampal cultures: the low affinity nerve growth factor receptor (p75/NGFR), HA, and CD8α. The distribution of HA was also examined after infection with a wild-type influenza virus, as in the initial experiments of Dotti and Simons (1990). p75/NGFR and HA are directly targeted to the apical surface in MDCK cells (Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1984; Le Bivic et al., 1991); CD8α is apically polarized at steady state but its delivery to the cell surface has not been examined (Migliaccio et al., 1990). These apical proteins did not display a polarized distribution when expressed in hippocampal neurons. Instead, significant cell surface expression was observed on both dendrites and axons (Figure 4; see also Craig et al., 1995). Staining appeared to extend throughout the entire extent of both the dendritic and axonal arbors. As with the basolateral sorting signal mutants that are apically targeted in polarized epithelia (mutant

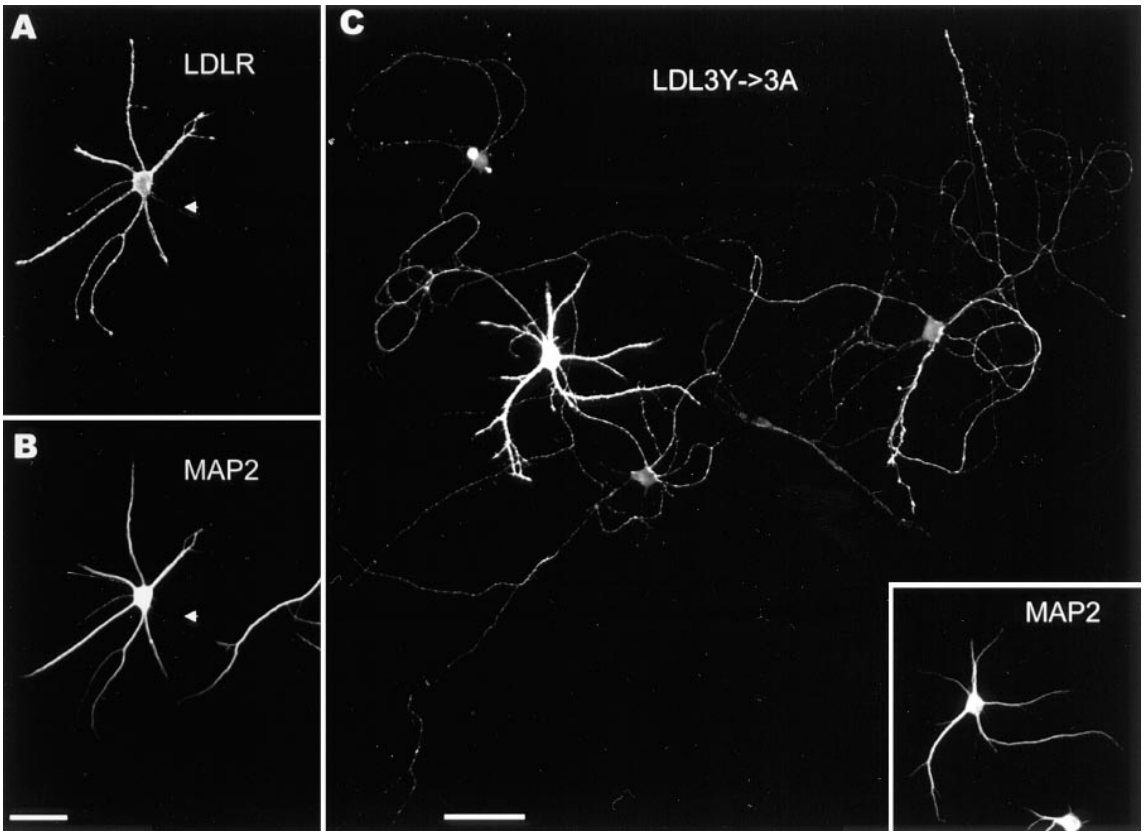

Figure 3. The Distribution of the Basolateral Protein LDLR and Its Basolateral Sorting Signal Mutant, LDLRY18,35,37 → AAA

Cell surface labeling of wild-type LDLR (A) was highly polarized to dendrites, as identified by MAP2 labeling (B). In contrast, cell surface labeling of mutant LDLR (C) extended throughout both the dendritic and axonal arbor. Labeling was most intense in the dendrites and the distal portions of the axon. The micrographs illustrate representative cells from 14-day-old cultures 18 hr after infection. Inset in (C), MAP2 labeling. Scale bar, 50 μm.

pIgR and LDLR), axonal staining was most intense distally. To determine whether the observed dendritic staining could be attributed to axons of labeled cells running along unlabeled dendrites, we double labeled influenza-infected cells with antibodies to HA and the endogenous axonal protein L1 (Figure 4). HA labeling was evident on the surface of L1-negative processes.

Table 1. The Degree of Polarization of Basolateral Proteins and Their Sorting Signal Mutants in Cultured Hippocampal Neurons

Protein	N	Hours Post Infection	% of Total on Dendritic Surface
TfR	15	18	98 ± 2
	10	36	98 ± 2
TfR 3-59	15	18	66 ± 11
	10	36	65 ± 12
pIgR	10	12	97 ± 3
	20	18	92 ± 6
	10	36	75 ± 8
pIgR 655–668	10	12	34 ± 11
	20	18	33 ± 10
	10	36	29 ± 13
LDLR	15	18	98 ± 2
	10	36	98 ± 2
LDLRY18 A	10	18	97 ± 3
LDLRCT33	10	18	96 ± 4
LDLRY18,35,37→AAA	10	18	54 ± 12
	5	36	56 ± 14

Values are mean ± SD.

The relative amount of axonal staining for these apical proteins varied between 50% and 71% (Table 2). Since the estimated surface area of the axonal arbor is 2–3.5 times greater than the dendrites, this suggests that the average concentration of these proteins is greater in dendrites than axons. As a more accurate estimate of the relative concentration of apical proteins in axons and dendrites, we measured the pixel intensity along line scans down the center of the axons and dendrites of HA-expressing neurons (Figure 5). The intensity of dendritic staining was 3–4 times that of staining along intermediate segments of the axon. Staining of the distal axon was about half that of dendrites, except at the axonal growth cone, which exhibited the brightest labeling of all. In contrast, staining of segments of the intermediate and distal axon of neurons expressing NgCAM via a defective AdV was on average 6-fold and 11-fold brighter than that of the longest dendrite (Figure 5).

We were concerned that the presence of apical proteins in dendrites might reflect missorting due to saturation of axonal sorting machinery. If so, higher levels of expression would be expected to yield more dendritic labeling. We therefore examined the relationship between level of expression (estimated by normalized total

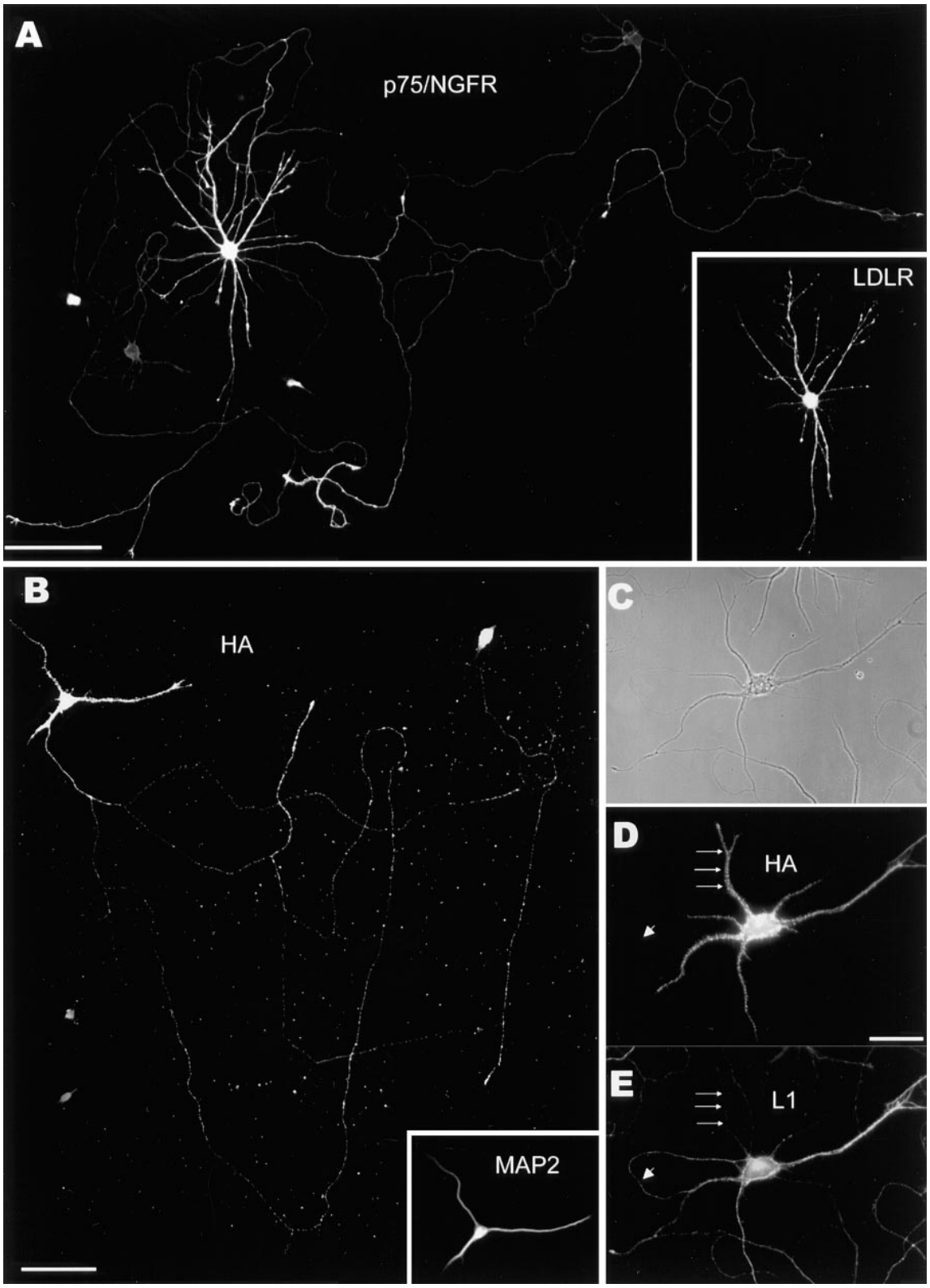

Figure 4. The Distribution of Two Apical Proteins, p75/NGFR and Influenza HA, following Expression in Hippocampal Neurons

In some cases, cells were coinfected with a virus expressing LDLR. (A) shows the distribution of p75/NGFR. Labeling was present throughout the dendrites and the axon; in contrast, cell surface expression of LDLR (inset) was polarized to the dendrites. Cell surface labeling of HA (B), expressed by infection with wild-type influenza virus (strain X:31 of Influenza A/aichi/2/68) also extended throughout the axon and dendrites (which were identified by MAP2 labeling, inset). Since influenza virus expresses HA in its coat, the fluorescent puncta that are not associated with neuronal processes most likely represent viral particles bound to the substrate. To test whether the dendritic labeling observed was due to axons running along dendrites, we double labeled influenza-infected neurons with antibodies to the endogenous axonal marker L1 (C–E). (C) is a phase micrograph illustrating the cell body, dendrites and proximal axon of such a cell. HA-labeled dendrites (D) that did not have axons running along them were observed (short arrows). In addition, some L1-labeled axons (E) that did run along HA-labeled dendrites were from other unlabeled neurons on the coverslip since they were not HA positive. The fluorescence micrographs of p75/NGFR and HA illustrate representative cells from 14-day-old cultures 18 and 12 hr after infection, respectively. Scale bar, 50 μm in (A) and (B); 20 μm in (D).

Table 2. The Degree of Polarization of Apical Proteins in Cultured Hippocampal Neurons

Protein	N	% of Total on Axonal Surface
P75/NGFR	10	71 ± 12
HA (herpesvirus)	10	57 ± 9
HA (influenza)	15	53 ± 10
CD8α	15	50 ± 10

fluorescence intensity per cell) and the extent of dendritic localization. Despite a 4-fold range in level of expression among different cells expressing a given construct, there was no significant correlation between level of expression and degree of polarization for any of the apical proteins (correlation coefficients ranged between −0.26 and 0.26).

Discussion

To test the hypothesis that neurons and epithelial cells use common cellular mechanisms for the sorting of polarized membrane proteins, we used replication-defective HSVs or AdVs to express basolateral proteins and apical proteins in 14-day-old hippocampal neurons in culture. Several aspects of our experimental strategy were novel. First, we used low viral titers to ensure that cells expressing the test constructs were well separated. Thus, interpretation of the distribution of the expressed protein could not be confounded by the axons of one labeled cell running along the dendrites of another. Second, we used fluorescence methods that allowed quantitative assessment of the relative amounts of the protein constructs present in each domain of the infected cells. Third, we demonstrated that species homologs of endogenous axonal and dendritic proteins were appropriately targeted following expression with these viral vectors. As predicted by the epithelial/neuronal hypothesis, basolateral proteins were targeted to the dendrites of hippocampal neurons and deletion or mutation of their basolateral targeting signals disrupted their dendritic targeting. Unexpectedly, apical proteins were not polarized to the axons of hippocampal neurons; they were distributed to both dendrites and axons in approximately equal amounts.

Parallels Between Basolateral and Dendritic Sorting

We found that three basolateral proteins, transferrin receptor (TfR), polyimmunoglobulin receptor (pIgR), and low density lipoprotein receptor (LDLR), were all targeted to dendrites. These results are consistent with previous findings that VSV G protein, SFV E protein, the betaine transporter, and the $GABA_A$ receptor are targeted to the basolateral surface of MDCK cells and the dendrites of hippocampal neurons (Rindler et al., 1984; Roman and Garoff, 1986; Dotti and Simons, 1990; Killisch et al., 1991; Perez-Velazquez and Angelides, 1993; Dotti et al., 1993; Ahn et al., 1996). As a more definitive test of the similarity between basolateral and dendritic protein targeting, we asked whether the same sorting signals were used for both. We found that mutations or deletions that disrupt the basolateral sorting of the TfR, pIgR, and LDLR also abolish their selective polarization to dendrites. This was particularly striking in the case of the LDLR, where point mutations of specific tyrosine residues changed the selectivity of targeting from 98% dendritic to only 55% dendritic.

In contrast to our observations with LDLR, recent work has shown that different sequences in the cytoplasmic tail of TfR mediate basolateral and dendritic targeting (Odorizzi and Trowbridge, 1997; West et al., 1997). West et al. (1997) found that dendritic sorting of TfR required the region surrounding a tyrosine essential for endocytosis, suggesting that it is the endocytosis signal itself that acts as a dendritic sorting signal for TfR. Our data demonstrate that endocytosis signals are not required for dendritic sorting, nor are they sufficient. The LDLR construct that contains an intact distal sorting signal (LDLRY18 → A) was polarized to the dendrites despite the fact that the proximal sorting signal, which also mediates its endocytosis, was mutated. Furthermore, the mutant pIgR 655–668 was not polarized to the dendrites, although its endocytic signal was intact.

Because the same sequences are active in both basolateral and dendritic sorting, the proteins that recognize this motif may also be similar in the two cell types. Given the similarity between basolateral sorting signals and endocytosis signals (Matter and Mellman, 1994; Mostov and Cardone, 1995; Le Gall et al., 1995), it is thought that sorting motifs are also recognized by adaptor proteins that recruit clathrin coats to induce vesicle formation (Boll et al., 1996; Heilker et al., 1996). Heilker and colleagues (1996) have shown that in vitro binding of AP1 and AP2 adaptors to peptides derived from hemagglutinin mutants correlates with their basolateral sorting. In addition, different tyrosine-based endocytic signals (including some that also act as basolateral targeting signals) bind with different affinities to AP1 and AP2 complexes, suggesting that the amino acid residues surrounding the critical tyrosine mediate interactions with specific adaptins, thereby sorting the protein into a unique vesicle (Boll et al., 1996; Ohno et al., 1996). It is unclear which adaptins are actually used for basolateral or dendritic sorting. In addition to AP1 and AP2, several novel adaptor-like proteins that could be involved in dendritic sorting have recently been discovered (Newman et al., 1995; Simpson et al., 1996; Dell'Angelica et al., 1997).

Two proteins appear to represent exceptions to the simple parallel between basolateral and dendritic targeting. One is Na^+K^+ATPase, which is endogenously expressed by both epithelial cells and neurons and is polarized to the basolateral domain of MDCK cells but is present in both the axons and dendrites of hippocampal neurons (Hammerton et al., 1991; Pietrini et al., 1992). Several lines of evidence, however, suggest that the mechanisms underlying the targeting of Na^+K^+ATPase differ from those of most other basolateral proteins. First, the targeting of Na^+K^+ATPase is disrupted by inhibition of sphingolipid synthesis, a treatment that does not interfere with the sorting of other basolateral proteins (Mays et al., 1995). Second, in some clones of MDCK cells Na^+K^+ATPase is not selectively targeted to the basolateral surfaces but accumulates there by selective stabilization; the selective targeting of other

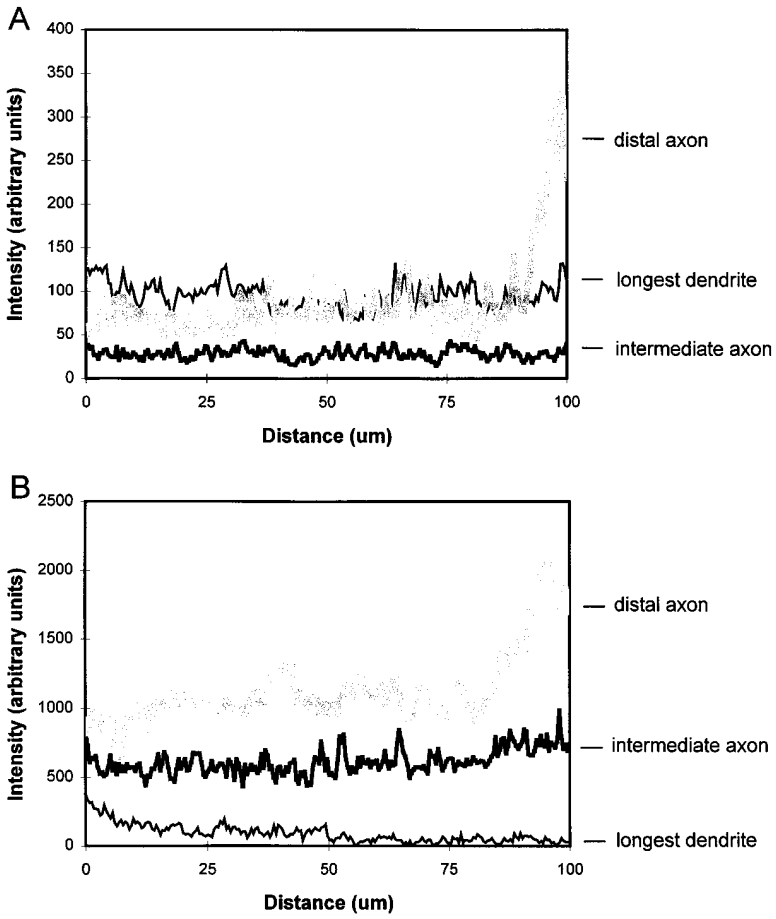

Figure 5. Comparison of the Intensity of Axonal and Dendritic Labeling in Cells Expressing HA or NgCAM

The intensity of dendritic staining was measured over the most proximal 50 μm segment and the most distal 50 μm segment of the longest dendrite of each cell. The intensity of axonal staining was measured along a segment 300–400 μm from the cell body (intermediate axon) and over the most distal 100 μm of the longest axonal branch (distal axon). HA staining was most intense in the dendrites and in the distal portion of the axon (especially at the axonal growth cone). The intensity of staining of intermediate portions of the axon was about 4-fold less than that of the dendrites. In contrast, NgCAM staining was much more intense in the axon than in the dendrites. The medial and distal segments of NgCAM-infected axons were on average 6- and 11-fold brighter than the longest dendrite. Labeling of the distal dendrites was at or near background. The intensity of staining was measured along the center of each of the aforementioned segments; values were normalized to the average pixel value in the longest dendrite. Each point represents the mean from 10 and 8 cells for HA and NgCAM, respectively.

basolateral proteins is not disrupted in these clones (Hammerton et al., 1991). β-amyloid precursor protein (β-APP) is a second possible exception to the parallel between basolateral and dendritic sorting. β-APP is targeted to the basolateral domain of MDCK cells, and its targeting depends on a cytoplasmic sorting signal containing a tyrosine motif (Haass et al., 1995), similar to those identified in many other basolateral proteins including LDLR (Matter and Mellman, 1994; Matter et al., 1994; Le Gall et al., 1995). In neurons, β-APP is initially targeted to axons via a glycosylation-dependent signal in its extracellular domain (Tienari et al., 1996) and subsequently becomes redistributed to dendrites (Simons et al., 1995; Yamazaki et al., 1995). This is consistent with the possibility that this protein contains both an axonal targeting signal, which is dominant in neurons, and a basolateral/dendritic signal that is effective in both cell types.

Is the dendritic polarization of basolateral proteins in neurons due to direct targeting or to a selective redistribution of proteins that are nonselectively inserted into the surfaces of both axons and dendrites? In epithelial cells, where the delivery of newly synthesized proteins can be assayed biochemically, all basolateral proteins that have been studied—with the single exception of Na^+K^+ATPase—are targeted directly to the basolateral surface. Whereas comparable methods cannot be applied to nerve cells, the kinetics of protein accumulation suggest that basolateral proteins are directly targeted to dendrites. This is most obvious in the case of the pIgR. At 12 hr after infection, when most neurons have been expressing detectable levels of protein on their surfaces for less than 2 hr, 97% of pIgR was already dendritic. If significant amounts of pIgR appear on the axonal surface before being redistributed to the dendrites, their residence time in the axonal membrane must be very short indeed. While we did not examine the kinetics of appearance of TfR or LDL-R as carefully, we observed nothing to suggest that the degree of their polarization increased over time.

The Localization of Apical Proteins in Neurons

We examined the distribution of five proteins whose apical targeting has been extensively studied in MDCK cells: p75/NGFR, HA, CD8α, and the sorting signal mutants of pIgR (pIgR 655–668) and LDL-R (LDLRY18,35, 37 → AAA). All five proteins were distributed in roughly equal amounts to axons and dendrites, a pattern of labeling that contrasts markedly from that observed when NgCAM, the chick homolog of the endogenous axonal protein L1, was expressed using a viral vector.

Previous reports that described the localization of two of these proteins (HA and pIgR 655–668) in cultured hippocampal neurons (Dotti and Simons, 1990; de Hoop et al., 1995; Ahn et al., 1996) concluded that these proteins were polarized to the axon. In each case, staining

was observed in dendrites as well as axons, but the dendritic staining was not viewed as significant. Following infection of hippocampal cultures with influenza virus, Dotti and Simons (1990) reported that 33% of HA-positive processes appeared to be dendrites. Ahn and colleagues (1996), who expressed HA by intracellular injection of an expression vector, also observed significant dendritic staining but attributed it to branches of the axon of the labeled cell that looped back along its own dendrites. In the case of pIgR 655–668 expressed using a Semliki Forest virus vector (de Hoop et al., 1995), dendritic staining was observed in 30% of neurons at early times after infection and in 80% of cells at a later time point. In this case, the dendritic labeling was attributed to missorting due to overexpression.

The experimental approach we have used to examine the distribution of expressed proteins in hippocampal neurons largely eliminates such confounding possibilities. By using low viral titers so that labeled neurons were widely separated and by comparing the distribution of expressed proteins with that of endogenous axonal markers, it was possible to eliminate the possibility that labeling associated with dendrites was due to the presence of fasciculating axons. Whereas it is difficult to exclude completely the possibility that the high expression levels that occur following virally mediated expression disrupt the targeting of apical proteins, four observations make this possibility unlikely. First, both endogenous dendritic proteins and exogenous basolateral proteins were highly polarized following expression with HSVs or AdVs. Second, NgCAM, the species homolog of the endogenous axonal protein L1, remained highly polarized when expressed at high levels using an AdV vector. Third, in the case of HA, we could compare directly the levels of expression with defective herpesvirus and wild-type influenza virus, the virus used in the initial experiments of Dotti and Simons, 1990. The expression with herpesvirus was about 30% less than with influenza virus; the extent of dendritic labeling in the two cases was virtually identical. Fourth, if the appearance of apical proteins in dendrites was due to saturation of axonal sorting machinery, higher levels of expression would be expected to yield more dendritic labeling. We observed absolutely no correlation between level of expression and the extent of polarization for any of the apical constructs.

Is it possible that apical proteins are initially targeted to the axonal surface but not retained there, resulting in the unpolarized distribution we observed? If this were the case, the percentage of ectopic, dendritic labeling would be expected to increase over time. We measured the distribution of pIgR 655–668 at 12, 18, and 36 hr after adding virus. At 12 hr after infection, when most infected neurons have been expressing detectable levels of protein on their surfaces for less than 2 hr, pIgR 655–668 was already present on the surfaces of dendrites and axons in approximately equal amounts. Its distribution did not change when expression was allowed to continue for an additional 6 or 24 hr. Likewise, none of the other apical constructs expressed showed a change in polarity between 18 and 36 hr after infection. This suggests that the dendritic localization observed for apical proteins was due to direct targeting. The distribution of another class of proteins, the GPI-anchored proteins, also appears to be at odds with the hypothesis that apical and axonal targeting employ a common mechanism. In MDCK cells, GPI-anchors are thought to act as apical targeting signals (Lisanti et al., 1989, 1990). Thy-1, an endogenous neuronal GPI-linked protein, has been reported to be polarized to the axon by some investigators (Dotti et al., 1991), but others have found it expressed in both dendrites and axons (Morris et al., 1985; Xue et al., 1990; Faivre-Sarrailh and Rougon, 1993). Another GPI-linked protein, F3/F11, was found to be axonal in cerebellar granule cells but unpolarized in Golgi neurons (Faivre-Sarrailh et al., 1992; Faivre-Sarrailh and Rougon, 1993). In contrast to F3/F11, three other endogenous, GPI-linked proteins are unpolarized in cerebellar granule neurons (Faivre-Sarrailh and Rougon, 1993). Likewise, expression of the exogenous GPI-linked proteins TIMP/Thy-1 (Lowenstein et al., 1994), CD8α-DAF, and axonin-1 (Burack, M. J., and G. B., unpublished data) via defective HSV or AdV resulted in labeling of both axons and dendrites.

Protein localization can be assessed with the least ambiguity in young cultures, before an extensive axonal network develops. At 1 or 2 days in culture, when endogenous axonal markers have begun to exhibit a polarized distribution (Fletcher et al., 1991; Van den Pol et al., 1993; Mandell and Banker, 1996), both HA and pIgR 655–668 have been reported to be unpolarized (Dotti and Simons, 1990), an observation that we have confirmed (M. J., Vogt, Sonderegger, and G. B., unpublished data). In contrast, endogenous L1 and virally expressed NgCAM were already well polarized at this stage of development (84% and 90% axonal; M. J., Vogt, Sonderegger, and G. B., unpublished data), although not to the same degree as in mature neurons.

Implications for Axonal Targeting

Our results demonstrate that the sorting information present in apical proteins is not sufficient to target these proteins to axons. This does not necessarily imply that neurons lack the machinery needed to recognize apical sorting signals or to sort apical proteins into a distinct class of transport vesicles. For example, in unpolarized cells, influenza HA appears to be sorted into a population of transport vesicles distinct from those containing the basolateral protein VSV G, but these vesicles are not targeted to distinct domains within the plasma membrane, as they are in polarized epithelia (Musch et al., 1996; Peranen et al., 1996; Yoshimori et al., 1996). Hence, it is possible that neurons also possess apical sorting machinery but do not selectively target "apical" transport vesicles to the axon.

Biochemical evidence also reveals differences in the sorting pathways of apical and axonal proteins. In MDCK cells, apical sorting is believed to involve glycosphingolipid-enriched membrane domains that provide sites for the clustering and immobilization of both GPI-anchored and other apical membrane proteins (van Meer and Simons, 1988; Brown and Rose, 1992; Hannan et al., 1993). As evidence of this, GPI-anchored proteins and transmembrane apical proteins, including influenza HA, aminopeptidase N, and sucrase-isomaltase, are associated with detergent-resistant complexes that are thought to

represent an intermediate compartment important for apical sorting (Skibbens et al., 1989; Danielsen, 1995; Mirre et al., 1996). In neurons, HA and several GPI-linked proteins are also associated with a detergent-insoluble fraction, but the axonally targeted protein β-APP is not (Tienari et al., 1996).

Because endogenous neuronal proteins such as NgCAM are selectively targeted to axons, they must contain sorting information that is not present in apical proteins. It will be important to identify the signals that underlie the sorting of axonal proteins and to determine at what point the trafficking pathways for apical and axonal proteins diverge.

Experimental Procedures

Cell Culture

Methods for preparing the hippocampal cell cultures have been described previously (Goslin and Banker, 1997). In brief, hippocampi were dissected from the brains of embryonic day 18 rats, and cell suspensions were prepared by trypsin treatment and trituration using a fire-polished Pasteur pipette. Cells were then plated onto acid-washed, poly-L-lysine–treated glass coverslips (Fisher, 18CIR-1 D, German glass, special order) in minimum essential medium (MEM) with 10% horse serum. After the neurons attached to the substrate, the coverslips were inverted and transferred into a dish containing a confluent monolayer of astroglia and were maintained in serum-free medium (MEM containing the N2 supplements of Bottenstein and Sato [1979], together with 0.1 mM sodium pyruvate and 0.1% ovalbumin). Small dots of paraffin on the coverslips supported them just above the glial monolayers (see Goslin and Banker, 1997).

Expression of Foreign Proteins in Primary Neuronal Cultures

Replication-defective HSV and AdV were used to express exogenous proteins in hippocampal neurons. We used the defective HSV system lacking the ICP4 gene product (Geller and Breakefield, 1988) in conjunction with the plasmid vector ("amplicon") developed by Wyborski et al. (1991) to package genes of interest, following the protocols described by Ho (1994). The amplicon contains the HSV-1 origin of replication and packaging site. The gene of interest was subcloned into the amplicon such that it was under control of the HSV-4 promoter, and then was transfected into E5 cells (a derivative line of Vero monkey kidney cells that expresses the HSV ICP4 gene product) with Transfectam (Gibco/BRL) concomitant with ICP4-minus replication-defective HSV. Viral stocks produced in this manner contain a mixture of defective helper virus and virions carrying tandem repeats of the amplicon. This mixture is eventually toxic to neurons in culture, but cells remain healthy for a window of about 48 hr (Lowenstein et al., 1994; Ho et al., 1995). The AdV-containing constructs were obtained from other laboratories and their preparation has been described previously. The AdV expression system is similar to the HSV system, except that the gene of interest is under the control of the CMV promoter and the resultant viral stock contains only the recombinant AdV (Becker et al., 1994). Toxicity due to AdV was not noted, even as long as 72 hr after infection.

Neurons were infected with defective HSV or AdV constructs, or wild-type influenza virus (strain X:31 of Influenza A/aichi/2/68, provided by Judy White, University of Virginia), by addition of an aliquot of viral stock directly to the medium in dishes containing neuronal coverslips. The amount of virus added was titered such that only 1%–5% of the neurons were infected. Kynurenic acid (500 μM) was added to cultures at the time of infection to lessen excitotoxic cell damage due to manipulation (Ho et al., 1995). In some experiments, sodium butyrate (5 mM) was added to enhance expression; this did not affect the distribution of expressed proteins.

Immunostaining

To detect virally expressed proteins present on the cell surface, living cultures were incubated with the primary antibody (diluted in glial-conditioned medium) for 5 min at 37°C. Cultures were then rinsed in phosphate buffered saline (PBS) and fixed in a solution of 4% paraformaldehyde/0.1% glutaraldehyde/4% sucrose in PBS. Cells were permeabilized by incubation in 0.25% Triton X-100 in PBS for 5 min (so they could be double labeled with antibodies to the dendritic marker MAP2) and then incubated in 10% bovine serum albumin (overnight at 4°C or for 2 hr at 37°C) in order to block nonspecific antibody-binding sites. Coverslips were then incubated with the appropriate biotinylated secondary antibody to detect the virally expressed protein together with either polyclonal or monoclonal antibodies to MAP2 (Binder et al., 1984), which were generously provided by Dr. Shelly Halpain, Scripps Institute, and Dr. Tony Frankfurter, University of Virginia, respectively. This was followed by incubation in FITC-conjugated streptavidin and the appropriate rhodamine- or Texas Red-conjugated secondary antibody to detect MAP2. Some cultures were also double stained with rabbit antisera to rat L1, an axonal marker (van den Pol and Kim, 1993), which was generously provided by Dr. Vance Lemmon, Case Western Reserve University (Drazba and Lemmon, 1990). Patterns of staining were qualitatively similar for the same constructs if live cells were incubated with primary antibody for only 2 min or if cells were fixed and not permeabilized prior to incubation with primary antibody.

Reagents

We would like to thank the following people who so generously gave cDNA, virus, and/or antibodies: Dr. James Casanova, Massachusetts General, wild-type and mutant pIgR cDNAs and pIgR sheep antisera (Casanova et al., 1991); Dr. Moses Chao, Cornell University, p75/NGFR AdV (Yoon et al., 1996) and p75/NGFR rabbit antisera (Huber and Chao, 1995); Dr. Robert Gerard, University of Texas-SWMC, LDLR AdV (Herz and Gerard, 1993); Dr. Joseph Goldstein, University of Texas-SWMC, LDLR rabbit antisera (Kowal et al., 1989); Dr. Vance Lemmon, Case-Western, NgCAM chick-specific monoclonal (Lemmon and McLoon, 1986); Dr. Ira Mellman, Yale University, mutant LDLR cDNAs (Matter et al., 1994); Dr. Peter Sonderegger, University of Zurich, NgCAM AdV (Vogt et al., 1996); Dr. Ian Trowbridge, Salk Institute, wild-type and mutant TfR cDNAs (Jing et al., 1990) and TfR human-specific monoclonal (Omary and Trowbridge, 1981); Dr. Judy White, University of Virginia, influenza HA cDNA (Ward and Dopheide, 1981) and influenza HA monoclonal (Kemble et al., 1992); Dr. Russell Wyborski, Parke-Davis, CD8α herpesvirus (Wyborski et al., 1991). Monocloanl antibodies against CD8α were purchased from Dako and monoclonal antibodies against LDLR were purchased from Amersham.

Quantitative Measurement of Immunofluorescence

Infected cells were chosen by examining fields at 2 mm intervals across the coverslip. A labeled cell whose processes traversed the field was selected for analysis, so long as its processes did not overlap those of other labeled cells; cells with fewer than three identifiable dendrites were also excluded. To limit possible photobleaching during the process of cell selection, total exposure time was kept to less than 10 s. In control experiments, this level of exposure was found to cause less than a 3% reduction in fluorescence intensity.

Images of immunofluorescently labeled cells ("specimen images") were acquired using a Photometrics CH250 chilled CCD camera (12 bit images, 1315 × 1017 pixels) and a Zeiss Axiophot (25× Planapo objective, NA 1.2). Exposure time was adjusted so maximum pixel value was at least half saturation. When acquiring the specimen image, the dark current image, generated by an equivalent exposure with the camera shutter closed, was subtracted.

After acquiring the specimen image, two additional corrections were performed. First a shading correction was applied to compensate for uneven illumination of the field (based on an image of a uniformly fluorescent field). Reliability of this shading correction procedure was confirmed by comparing images of fluorescent beads (MultiSpeck Slides, Molecular Probes) with and without shading correction. Second, the average background staining of uninfected cells was subtracted. Because dendrites are thicker than axons, the background staining of dendrites was greater than that of axons; separate values for the average background intensity were measured for dendrites (MAP2 positive) and axons (MAP2 negative).

The perimeters of processes from the corrected specimen image were traced using aligned phase images or aligned MAP2-labeled

images. Processes labeled with MAP2 were considered dendrites, and MAP2-negative processes were considered axons. In those cases where the expressed protein was confined to the soma and dendrites, the axon of the infected cell often could not be distinguished unambiguously from the axons of neighboring, uninfected cells. In this case, the fluorescence associated with the brightest MAP2-negative process was measured to provide a maximal estimate for axonal labeling. The total pixel intensity of all of the dendrites of a cell was summed and compared to the total pixel intensity measured in the axon, providing a measure of the relative amount of expressed protein present in the two compartments. The total pixel intensity of all the dendrites and the axon was summed providing a measure of the relative amount of expressed protein on the cell surface. The relationship between the total pixel intensity of a neuron and the extent of its polarization was examined using the Pearson Product Moment Correlation Test. Values for the degree of polarization of each construct are based on analysis of 10–20 cells chosen from two to four separate culture preparations. Intensity measurements were based on line scans along the center of the longest dendrite and the axon, which was measured at an intermediate distance from the soma (300–400 μm) and at the tip of the longest branch.

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