An intrinsic compartmentalization code for peripheral membrane proteins in photoreceptor neurons

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In neurons, peripheral membrane proteins are enriched in subcellular compartments, where they play key roles, including transducing and transmitting information. However, little is known about the mechanisms underlying their compartmentalization. To explore the roles of hydrophobic and electrostatic interactions, we engineered probes consisting of lipidation motifs attached to fluorescent proteins by variously charged linkers and expressed them in Xenopus rod photoreceptors. Quantitative live cell imaging showed dramatic differences in distributions and dynamics of the probes, including presynapse and ciliary OS enrichment, depending on lipid moiety and protein surface charge. Opposing extant models of ciliary enrichment, most probes were weakly membrane bound and diffused through the connecting cilium without lipid binding chaperone protein interactions. A diffusion-binding-transport model showed that ciliary enrichment of a rhodopsin kinase probe occurs via recycling as it perpetually leaks out of the ciliary OS. The model accounts for weak membrane binding of peripheral membrane proteins and a leaky connecting cilium diffusion barrier.

Introduction

Neurons are highly polarized cells with distinct domains that receive and transmit signals. Proper function of neurons thus relies on correct distribution of proteins into functional compartments. Defects in protein trafficking are associated with devastating neurodegeneration or dysfunction. The mechanisms underlying the differential distribution of proteins in neurons are, thus, of fundamental importance. Trafficking of intrinsic membrane proteins has been well studied (reviewed in Bentley and Banker, 2016). How compartmentalization of peripheral membrane proteins (PMPs) is achieved, however, is poorly understood.

Posttranslational lipid modifications have emerged as an important mechanism for PMP localization to specific compartments (El-Husseini et al., 2000; Kerov et al., 2007). Three main types of protein lipidation are myristoylation, prenylation, and palmitoylation. In neuronal protein trafficking, the role of reversible protein palmitoylation is the most well characterized (reviewed in Fukata and Fukata, 2010). Less is known about the roles of N-terminal acylation and prenylation, irreversible forms of lipidation, in targeting PMPs to their functional compartments. In vitro, prenyl and acyl lipid modifications impart relatively weak membrane association on their own (Peitzsch and McLaughlin, 1993). Other properties of PMPs, including surface charge and/or association with other intrinsic cell structures, are needed to provide significant membrane association (Hancock et al., 1990; Murray et al., 1997; Resh, 2016). However, it is not known if acyl or prenyl moieties and surface charge are sufficient, on their own, to produce differential compartmentalization of PMPs in living neurons, or how this localization occurs.

In this study we addressed the roles of prenylation, acylation, and electrostatic charge on the subcellular enrichment and dynamics of PMPs in a sensory neuron, the retinal rod photoreceptor. Rods have three main compartments—the ciliary outer segment (OS), which is packed with membranous discs and phototransduction proteins, the presynaptic spherule, and the cell body. Targeting of some intrinsic membrane proteins to the OS and cell body has been addressed (Baker et al., 2008; Deretic and Wang, 2012; Lodowski et al., 2013; Wang and Deretic, 2014). However, aside from several studies examining the roles of lipid binding chaperone (LBC) proteins (Zhang et al., 2011, 2012; Thomas et al., 2014; Hanke-Gogokhia et al., 2016; Rainy et al.,...
the mechanisms underlying the subcellular localization of PMPs to specific rod compartments are not known. Here we show that lipid modification and nearby electrostatic charge alone result in dramatic differential distribution of PMPs, and we propose a mechanism for regulating their enrichment within, or depletion from, photoreceptor cilia. Lipid moiety and surface charge thus constitute an intrinsic compartmentalization code for PMPs in photoreceptors.

**Results**

**Lipid modification and surface charge alone lead to compartment enrichment of PMPs in rods**

To evaluate the roles of lipid modification and protein surface charge on PMP distribution in rods, we engineered fluorescent protein probes consisting of short peptides containing different lipidation motifs and charged or neutral amino acids, fused to EGFP or its photoactivatable variant, PAGFP (Fig. 1 A). The probes did not contain known binding motifs for phototransduction or LBC proteins so that the distribution patterns observed upon expression in *Xenopus laevis* rods would be the result of probe electrostatic and hydrophobic interactions with cell structures. Three probes derived from endogenous lipitated proteins, the α and γ subunits of the G protein supporting phototransduction, transducin (Tα and Tγ), and rhodopsin kinase (GRK1), the G protein receptor kinase involved in halting phototransduction, were generated as well. Distributions of the probes were evaluated by confocal imaging of live retinal explant slices (Fig. 1, B and C). Fig. 1 D shows the structures of the lipid moieties appended to the probes and estimates of their membrane binding energies and dissociation constants.

Remarkably, the probes displayed highly divergent distribution patterns among the rod compartments (Fig. 2, A and B). Relative enrichment of the probes within the two major functional compartments, the ciliary OS and the synapse, were quantified next (Fig. 2 C). Non-lipidated, neutrally charged probe (NL0) served as a benchmark, accounting for effects of protein size and steric interactions (Najafi et al., 2012). Positive charge-bearing prenylated probes were enriched within the synapse to a degree that depended on charge magnitude, with enrichment of +8 probes reaching >10-fold. Acylated probes, on the other hand, showed no significant difference in the synapse/OS ratio, regardless of linker charge (Fig. 2 C). Controls showed that probes with charged linkers and no lipid did not result in synaptic or OS enrichment (Figs. 2 B and 3 A) and that the addition of 21 amino acid linkers to C or N termini of EGFP did not impact the distribution (Fig. S1). Dramatic differences in the enrichment of PMPs between the two major functional rod compartments thus appeared to be encoded by lipid modification and protein surface charge alone.

**Synaptic enrichment of positively charged prenylated probes is the result of OS depletion**

To obtain a finer grain view of the differences in compartment enrichment, we evaluated probe distribution profiles within five subcellular domains: the synapse, nucleus, myoid, ellipsoid, and OS (Fig. 3). NL0 was broadly distributed throughout the cell, with the synaptic, nucleus, and myoid regions approximately uniformly filled and the ellipsoid and OSs containing twofold or lower fluorescence (Fig. 3 A), in agreement with our previous results (Peet et al., 2004; Calvert et al., 2010; Najafi et al., 2012). The distributions of the prenylated probes, on the other hand, were not uniform (Fig. 3, B and C). The concentration of prenyl probes was significantly lower in the nucleus than in any of the other cell body/inner segment (IS) compartments, a result that holds for all the lipidated probes in this study. Importantly, the distribution patterns did not change over at least a decade of expression (Fig. S2), indicating that differences were not due to saturation of binding or other nonspecific interactions.

The OS concentrations of prenylated probes with positively charged linkers were more than fivefold lower than the IS concentrations (Fig. 3 B). The concentration in the synapse was slightly higher on average than the myoid or ellipsoid. The neutral or negatively charged prenylated probes were present in the OS at higher concentrations (Fig. 3 C). Neutral probes often appeared in a concentration gradient from OS base to tip, and synaptic enrichment was less prominent.

To quantify the differences in distribution among the major rod compartments, we chose to compare the concentrations of the probes relative to the myoid, which serves as the protein synthesis and processing center of rods (Fig. 3, D and E). We refer to the concentration ratios as the OS (OSEI) or synapse enrichment indices (SynEI). Remarkably, positive prenyl probe OSEIs were seven-, five-, and threefold below that of NL0 (Fig. 3 D) and thus were depleted from the OS.

Nearly the opposite pattern was found for synaptic enrichment. SynEIs for positively charged prenyl probes were ~1.6-fold greater for the +8 probes and 1.2-fold for the FarΔ+4 probe, values that were significantly different from NL0. SynEIs for negatively charged probes were not significantly different from NL0 (Fig. 3 E). Of note, the magnitude of the SynEIs, maximally 1.6, was significantly below the up to sevenfold OS depletion. These results show that the >10-fold enrichment of positively charged prenylated probes in the presynapse relative to the OS was mostly due to depletion of the probes from the OS.

**Positively charged prenyl probes are enriched in the IS plasma membrane, apical membrane region, and perinuclear membrane**

In addition to enrichment in the synaptic sphere, prenyl +8 probes appeared to be enriched in multiple IS membrane domains, including the plasma membrane and perinuclear membranes (Fig. 4 A). Fluorescence signal from the plasma membrane of the OS, however, was notably absent.

Curiously, the region of the OS–IS junction appeared to be particularly enriched with the +8 prenyl probes (Fig. 4 A, arrowheads). We wondered if this enrichment indicated that the apical membrane of the IS compartment served as a sink for +8 prenyl proteins, which might be involved in the OS depletion mechanism. The OS–IS junction, however, is a densely packed structure with apical membrane, connecting cilium, calycal processes (CPs), nascent discs, mitochondria, and mature discs (Fig. 2 A), all within a few hundred nanometers. With a resolution limit of ~240 nm, confocal microscopy does not allow...
absolute identification of which of these structures contain the probes; however, closer examination gives some important clues. 3D image stacks show that CPs are fluorescent (Fig. 4 B, arrowheads; Videos 1, 2, and 3), an observation bolstered by examining broken OS–IS where some of the CPs are displaced (Fig. 4 C). This shows that some of the OS–IS junction signal comes from the CPs that are contiguous with the apical membrane. To evaluate if the OS–IS junction signal represented a
structure larger than the apical membrane, we analyzed the OS–IS enrichment bandwidth (Fig. 4 D). Intensity profiles of rods expressing Far+8 and GG+8 probes were collected along a computer-generated line that extended axially, through the 3D center of the photoreceptor (Fig. 4 D). On average, the full width at half maximal (FWHM) fluorescence was 726 ± 113 nm for Far+8 and 970 ± 84 nm for GG+8, more than threefold larger than the FWHM of the point spread function (psf) intensity.
profile, thus showing that the fluorescence signal at the OS–IS junction cannot come from the apical membrane alone.

No structure in the OS–IS junction region, except for the nascent disc membranes that form at the OS base and the fully formed discs enclosed within the OS plasma membrane possess such a flattened shape. Thus, the prenyl +8 probes likely enter the connecting cilium compartment and fill the nascent discs, which extend ~100–300 nm above the apical membrane (Besharse et al., 1977)—and likely some of the isolated discs within the OS plasma membrane. Further evidence that small amounts of the probe are present in the OS discs comes from analysis of the fluorescence distribution along the full length of the OS (Fig. 4 E). The fluorescence signal is higher proximal to the OS–IS junction and tapers downward along ~10 µm of the OS (Fig. 4 E, left panels). This pattern is clearly distinct from that of NL0 and other lipidated probes, where the OS distribution is more uniform (Fig. 4 E, right panels).

It was surprising that +8 prenyl probes were enriched in discs at the base of the OS and do not reach more distal discs. Physically, there are few possible mechanistic explanations: +8 prenyl probes either have a nonuniform pattern of relatively high-affinity binding sites in the nascent and basal OS discs, or there is an active mechanism removing the probe from the OS. To distinguish between these possibilities, we examined the mobility of the +8 prenyl probes in the OS–IS junction and

Figure 3. Distribution of prenylated probes depends on linker charge. (A–C) Upper panels, representative confocal images. Scale bars, 10 µm. Lower panels, average compartment fluorescence normalized to total cell fluorescence. (D) OSEI, defined as the ratio of average OS fluorescence to average myoid fluorescence, F_{OS}/F_{M}. (E) SynEI, defined as ratio of average synaptic fluorescence to average myoid fluorescence, F_{syn}/F_{M}. (D and E) Lower panels are significance tables. *, P = 0.05; NS, not significant. Box fills as in Fig. 2 C key. Box-whisker plots as described in Fig. 2. n for each construct: NL+8, 14; NL0, 13; NL-8, 17; GG+8, 14; GG0, 20; GG-8, 22; Far+8, 13; Far+4, 21; Far0, 42; and Far-8, 16.
We reasoned that if the probes were localized to the basal discs due to high-affinity binding, then the probe found in the OS–IS junction would be poorly exchangeable with probe in other regions of the cell body. We found that the synaptic region rapidly recovered after photobleaching, with a $t_{1/2}$ of $\sim 2$ min, and that this recovery was mirrored by a decline in signal at the OS–IS junction (Fig. 4, F and G). Thus, Far+8 at the OS–IS junction and the synaptic region are rapidly exchangeable, and binding to these structures is relatively weak.

Distribution and transport of prenylated probes does not require association with the prenyl binding/chaperone protein, PrBPδ

The prenyl binding protein, PrBPδ, has been implicated in the OS transport and localization of PDE6αβ subunits (Zhang et al., 2007; Hanke-Gogokhia et al., 2016; Wright et al., 2016) and GRK1 (Zhang et al., 2007), all of which are prenylated PMPs. We thus explored the possibility that PrBPδ binding to the prenylated probes solubilizes them for transport across the connecting cilium diffusion barrier. A GFP trap assay, however, failed to pull down PrBPδ with any of the engineered prenyl probes examined, despite identifying it in the input and flow-through (Fig. 5, A–C). We next examined the distribution and PrBPδ association of a probe containing the 18 N-terminal amino acids of GRK1. Interestingly, the OS enrichment index of $\sim 4.5$ for EGFP-GRK1ct18 shows that it was highly enriched in the OS compartment (Fig. 5, D and F). The OS enrichment of full-length GRK1 has not previously been determined. However, our analysis of immunohistochemical staining of GRK1 in mammalian photoreceptors.
showed a range of two to four for the OSEI, in reasonable agreement with the enrichment of EGFP-GRK1ct18. Our results thus show that the N-terminal 18 amino acids of GRK1 are sufficient to encode GRK1 OS enrichment.

GFP trap of EGFP-GRK1ct18 quantitatively pulled down endogenous X. laevis homologue of PrBPδ (Fig. 5 G). In contrast, a farnesylated probe containing the 19 N-terminal amino acids of transducin γ, EGFP-Tγct19, like the other engineered Far probes, failed to pull down endogenous PrBPδ (Fig. 5, A–C and H), and its distribution was not different from NL0 (Fig. 5, E and F), in agreement with previous reports in PrBPδ knockout mouse (Zhang et al., 2007). These results directly show that PrBPδ interaction with GRK1 is necessary for its OS enrichment. However, PrBPδ is not necessary for prenylated proteins to gain access to the ciliary OS compartment.

Acylation leads to probe enrichment within the OS independent of linker charge or association with Unc119

We next examined the enrichment of acylated probes in the OS and synapse compartments relative to the myoid (Fig. 6). All Myr probes were significantly enriched in the OS, relative to NLO, regardless of linker charge (Fig. 6, A and C). Myr0 had the highest OSEI at 2, and Myr+8 and Myr-8 were approximately equally OS enriched with OSEI ~1; NLO OSEI was 0.45 (compare Fig. 3, A and D). The distribution of ntl6Tα-EGFP was not significantly different from Myr+8 or Myr-8 (Fig. 6, B and C). Myr0 had a significantly higher OSEI than Myr+8, Myr-8, and ntl6Tα-EGFP (Fig. 6 C).

Except for Myr+8, the SynEIs for all Myr probes was ~0.75, not different from NLO (Fig. 6 D). At ~1.6, Myr+8 SynEI was significantly higher, showing that positive charge on a myristoylated PMP can lead to synapse enrichment. However, none showed significant preference between the major rod compartments (OS and synapse; Fig. 2 C). This result shows that in order for myristoylated PMPs to be enriched in one or other of the major functional compartments, an active process of enrichment or depletion of probe would be required, as observed with the prenyl probes.

Previous studies have implicated an acyl binding protein, uncoordinated 119 (Unc119), in the transport and OS enrichment of Tα (Zhang et al., 2011). We were somewhat surprised that the ntl6Tα probe was not enriched in the OS beyond the generic Myr probes (Fig. 6, B and C). Thus, we wondered whether the Unc119 mechanism was operating in frog rods. A search of Xenbase showed that the X. laevis genome possesses two homologues, one to Unc119 and the other to Unc119b (Xenbase gene IDs: XB-GENE-6487917 and XB-GENE-17343734, respectively), with ~80% homology to mouse Unc119. However, Western blots of X. laevis retina extract with three Unc119 antibodies failed to identify xUnc119, showing that either it was not expressed in retina or the antibodies did not cross-react. To distinguish between these possibilities, we performed mass spectrometry protein identification on the 25–30 kD MW region of retinal
extract run on SDS PAGE. xUnc119 was identified in this band, showing that it is indeed expressed in X. laevis retina (Table S1).

We examined GFP trap pulldowns of probes with mass spectrometry protein identification. GFP trap from retinas expressing Myr0-EGFP failed to pull down xUnc119 (Table S2), while xUnc119 was identified in nt16Tα-EGFP pulldowns (Table S3). Thus, Myr0 probe was not a significant binding partner for Unc119. Importantly, the mass spec analysis of the Myr0-EGFP probe GFP trap eluate contained ∼1,500 different proteins, from virtually every membrane compartment, including synaptic vesicles, IS plasma membrane, OS plasma membrane, mitochondria, lysosomes, and disc membranes. Despite this rich trove of proteins, no other known or putative LBC proteins were identified (Table S2). Thus, acylated probes enter and become enriched within the OS without Unc119 interactions, in agreement with Unc119 knockout studies in mice (Zhang et al., 2011), and without interactions with other LBCs. Moreover, the lack of OS localization of the nt16Tα-EGFP probe directly shows that Unc119 association alone is not sufficient for strong OS enrichment of Tα.

Binding affinity to disc membranes is not sufficient for localizing PMPs to the OS
Extant models for ciliary enrichment of lipidated PMPs suggest that LBC proteins solubilize the PMPs, allowing them to diffuse in the cytoplasm between ER/Golgi and carrier vesicles, or to diffuse directly into the ciliary compartment, where they are then released onto the vesicle or disc membranes (Hanke-Gogokhia et al., 2016; Wright et al., 2016). These models assume that the PMPs tightly bind to target membranes and that the localization is the result of local binding sinks. While such mechanisms are consistent with LBC knockout experiments (Zhang et al., 2007; Hanke-Gogokhia et al., 2016; Wright et al., 2016), the central premise of the mechanism, tight membrane binding, has not been directly tested. We reasoned that because the rod OS disc membranes are isolated from the plasma membrane, and from each other, the mobility along the length of the OS compartment would be a direct measure of the affinity of the probe for disc membrane. Thus, to test the idea, one need only examine the mobility of the probes along the OS compartment length and compare them to the mobility of unmodified E/PAGFP. The geometry of the OS makes it an ideal, and perhaps the only in vivo model for assessing the ciliary membrane affinity of PMPs.

PAGFP or EGFP probes expressed in rods were photoactivated or photobleached in patches of arbitrary size along the OS length, and the redistribution was followed over time (Fig. 7, A and B). To quantify the mobilities of the probes, we fitted the relaxation time courses to a bounded diffusion model (Theory, see Calvert et al., 2010). Diffusion coefficients along the length of the OS, $D_{OS}$, were estimated by root mean square error minimization (Fig. 7, B and C). Surprisingly, $D_{OS}$ of GRKctl5-EGFP

Figure 6. Myristoylated probes are enriched in the OS regardless of linker charge. (A) Upper panels: Representative confocal images of rods expressing Myr probes. Lower panels: Compartment distribution profiles. (B) Confocal image and compartment distribution profile of Tant16. (A and B) Scale bars, 10 µm. (C) OSEIs of myristoylated probes. Box-whisker plots as described in Fig 2. Significance table shows that all probes had significantly higher OSEIs than NL0. (D) SynEIs show that Myr+8 is the only presynapse enriched probe. Significance tables: P ≤ 0.05; NS, not significant. n for each construct: Myr+8, 27; Myr0, 28; Myr-8, 15; and Tant16, 18.
was not significantly different from that of Far0, and only approximately twofold lower than unmodified E/PAGFP (Fig. 7 D). Thus, GRKct15 binds to disc membranes with approximately the same low affinity as Far0, a probe that does not localize to the OS.

The affinity of Myr probes for the OS disc membranes differed up to fourfold depending on linker charge, with the Myr8+ having the highest affinity, and thus the lowest $D_{OS}$ and Myr8− the lowest affinity and the highest $D_{OS}$ (Fig. 7 D). The OSEI did not, however, follow the disc affinities of the probes. Myr0 was significantly more OS enriched than either Myr+8 or Myr−8, which were enriched to approximately the same extent. Thus, OS enrichment of acylated probes did not appear to result from affinity to disc membranes.

Previous studies in mammals have shown that the myristoylation motif found on Tα leads to heterogeneous acylation (mostly C14:2 and C14:1/C12:0) (Kokame et al., 1992; Neubert et al., 1992; Johnson et al., 1994; Neubert and Hurley, 1998; Lobanova et al., 2007), although in frogs Tα possessed C14:2 only. Among these lipids, C14:2 has the lowest affinity for membranes, on par with that of Far0 (Peitzsch and McLaughlin, 1993; Silvius and l’Heureux, 1994). C14:1 and C12:0 have equal, approximately fivefold higher affinity than C12:2. The affinity of C14:0 is fivefold higher again. $D_{OS}$ for Myr0 in our study was approximately four- to fivefold lower than that of Far0 (Fig. 7 D), and the relaxation of the photoconversion field in FRAP experiments was well fitted assuming a single diffusion coefficient. Thus, the Myr probes in our study were predominantly modified with C14:1 and/or C12:0 acyl lipids.

**The connecting cilium is a leaky barrier to PMP diffusion**

An alternative to the tight binding sink mechanism for retaining PMPs in the OS may be restriction of movement through the connecting cilium by means of a diffusion barrier. Diffusion barriers for intrinsic membrane and soluble proteins at cilia transition zones have been proposed by several groups (Sorokin, 1962; Spencer et al., 1988; Vieira et al., 2006; Geneva and Calvert, 2006).
cilium (Najafi et al., 2012) and it was later shown that the ciliary OS, and their equilibration throughout the rod was estimated, so the effective DCC reflects any tortuosity, other properties of the axoneme, or other ciliary contents that may impact free diffusion.

nFlux was time invariant for all of the engineered lipidated probes we analyzed (Fig. 8 D), showing that their transport through the connecting cilium was via simple diffusion. DCC of PAGFP-0Far, Myr-8-PAGFP, and Myr0-PAGFP were 10-fold lower than that of unmodified PAGFP (Fig. 8 E), indicating that the permeability of the connecting cilium to the lipidated probes was significantly lower than that of the unmodified PAGFP. This difference cannot be attributed to binding of probes to OS disc membranes alone since DOS of Far0 and Myr-8 were only approximately twofold lower than that of PAGFP (Fig. 7). Note that the DOS was invariably lower than DCC for all probes measured. This is due to the high degree of tortuosity of the diffusion paths imposed by the disc membranes (Calvert et al., 2010). Thus, the connecting cilium impedes but does not prevent the intercompartment diffusion of lipidated proteins.

**Diffusion-binding-active transport (DBT) model of subcellular enrichment of PMPs**

Thus far our results have revealed that lipidated proteins have varied affinities for disc membranes, their transport through the connecting cilium is impeded, and their distributions depend on both the lipid moiety and linker charge. How these factors come together to produce the various steady-state distributions, however, is not clear. In this section we use a DBT model to explore the impact of measured parameters on the steady-state distributions of PMPs.

The DBT model (Eq. 2; and Theory Eqs. A13, A14, and A15) allows computational evaluation of the impact of spatially variable diffusivity, local binding, and local active transport on the dynamics and steady-state distributions of molecules in polarized cells.

\[
\frac{dM_{IS}}{dt} = nFlux = D_{CC} \frac{A_c}{l_{cc}},
\]

where \(dM_{IS}/dt\) is the flux of molecules from the OS, through the connecting cilium and into the IS, \([c_{OS}(t) - c_{IS}(t)]\) is the time varying concentration gradient, \(D_{CC}\) is the effective diffusion coefficient through the connecting cilium for the molecular species, and \(\frac{A_c}{l_{cc}}\) is the ratio of the area of cross-section, \(A_c\) and the length of the connecting cilium, \(l_{cc}\). nFlux is time invariant if transport is via simple diffusion. The magnitude of \(D_{CC}\) may then be obtained based on measured nFlux and estimates of \(A_c\) and \(l_{cc}\) from published EM studies (Calvert et al., 2010). The entire cross-section of the CC is used in \(A_c\) estimation, so the effective \(D_{CC}\) calculation takes into account any tortuosity, other properties of the axoneme, or other ciliary contents that may impact free diffusion.

**Rearrangement of Fick’s first law yields a normalized flux term, nFlux,**

\[
\frac{1}{1 + \frac{B(\varepsilon)K_c(\varepsilon)}{(K_c(\varepsilon) + \varepsilon)}} \frac{d}{\partial z} \left( \frac{D(z)A(z)\frac{\partial c_f}{\partial z}}{A(z)} \right) = \frac{1}{\partial z} \left( A(z)D(z)\frac{\partial c_f}{\partial z} \right)
\]

Eq. 2 consists of three terms representing local binding.
and active transport, 

\[ \frac{1}{A(z)} \frac{\partial}{\partial z} \left( A(z) D \frac{\partial c}{\partial z} \right) \].

To reduce computational burden, we assume that the cell is approximately cylindrical and that transport of molecules along the shorter radius of the cell is fast relative to transport along the longer axis (see rod schematic, Fig. 2 A). This allows reduction of the model to a single spatial dimension, except that the area of cross-section varies with distance. To account for this, an axially variable area of cross-section, \( A(z) \), was introduced. As the binding term shows, local binding is assumed to operate according to a Langmuir isotherm. The synaptic spherule and distal tip of the OS are treated as no-flux boundaries (Theory Eq. A14), i.e., molecules cannot leave the cell. Finally, we assume the concentrations of molecules remain constant over the time required for equilibrium distribution to be achieved, i.e., no molecules are being synthesized or degraded. Model variables and their units are given in Table 1.

We used the model to examine EGFP-Far0 and EGFP-GRK1ct18 probes because of the striking difference in their steady-state distributions despite similar \( D_{OS} \) and linker charge. The major difference between these probes is that EGFP-GRK1ct18 binds with PrBP, whereas EGFP-Far0 does not. PrBP has been shown to distribute throughout the cell body, but was absent from the rod OS in frog rods (Norton et al., 2005). The modest reduction of the \( D_{OS} \) from E/PAGFP suggests the membrane binding affinity is low for these probes, but how low binding affinity, particularly if it is not spatially uniform, impacts distribution is not clear. We thus initially explored if modest affinity binding in the OS, relative to the cell body, could lead to the OS enrichment observed for EGFP-GRK1ct18 (Fig. 9, A–C).

We estimated the binding affinities of the probes by finding the capacity, \( B \), and the dissociation constant, \( K_d \), in the diffusion-binding model that reduced the apparent OS diffusion coefficient twofold. We then introduced the observed 10-fold diffusion impediment to PMPs traversing the connecting cilium in the full DBT model and allowed it to run until a steady-state distribution was achieved. We found that a variety of combinations of \( K_d \) and \( B \) could produce the twofold changes in the effective \( D_{OS} \) with similar changes in OSEI; thus, we present the results in terms of equivalent binding power (EBP), where \( EBP = K_d / B \). An EBP of 1.5 resulted in twofold reduction in effective \( D_{OS} \) but produced an OSEI of only \( \sim 0.55 \), slightly higher than unmodified E/PAGFP but not close to the OSEI of approximately five observed for EGFP-GRK1ct18 (Fig. 9, B, C, and F). Higher OSEI were obtained with higher affinity OS binding, but at the cost of dramatically reduced OS mobility (Fig. 9 F).

Introducing active transport within the connecting cilium with an OS-directed transport velocity, \( v = 0.55 \) \( \mu \text{m s}^{-1} \) combined with the weak OS binding resulted in OSEI of \( \sim 0.8 \) (Fig. 9, D and F). To put this velocity into perspective, given that GRK1 is present in the rod at a ratio of 1:800 rhodopsins (Klenchin et al., 1995), which corresponds to a cytoplasmic concentration of \( \sim 3.75 \) \( \mu \text{M} \), \( v = 0.55 \) \( \mu \text{m s}^{-1} \) is equivalent to transporting \( \sim 17 \) molecules of GRK1 per second through the axoneme-excluded CC. Finally, adding a CC diffusion impediment, reducing \( D_{cc} \) to 0.4 \( \mu \text{m}^2 \text{s}^{-1} \), was necessary to achieve the OSEI of approximately five (Fig. 9, E–G). Active transport of some form and impeded connecting cilium diffusion thus appear to be the major factors needed to produce the observed OS enrichment of EGFP-GRK1ct18, and by extension, enrichment of GRK1.

Finally, we examined whether a weak local binding sink could account for the degree of synapse enrichment and the rapid exchange among IS pools found for Far+8 (Fig. 9, H–j). Indeed, a \( K_d \) of 0.5 \( \mu \text{M} \) in the synapse was sufficient to produce the observed SynEI, with a \( t_{1/2} \) of FRAPb recovery of \( \sim 2 \) min, as observed for Far+8.

**Discussion**

Extant models for the transport and confinement of PMPs to subcellular compartments include vesicular transport, transport by motor-driven protein complexes, and diffusion, all of which are mediated by LBC proteins (Baehr, 2014; Jensen and Leroux, 2017). All of these theories involve tight membrane binding or a selective diffusion barrier to maintain steep concentration gradients, i.e., they either implicitly or explicitly hypothesize that once a PMP is delivered to a compartment, it remains there indefinitely.

Our results do not fit this model. Weak membrane binding of PMPs in our study shows that they would not remain on their target membranes for more than a few minutes. The DBT model predicted that moderate binding within the synaptic spherule could lead to significant synapse enrichment, while maintaining rapid exchangeability with other cell body membranes. However, neither the weak binding to disc membranes nor the modest impediment to diffusion imparted by the connecting cilium could explain PMP enrichment within the ciliary OS.

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**Table 1. DBT model variables and units**

| Variables | Description | Units |
|-----------|-------------|-------|
| \( c_f \) | Concentration of unbound molecules | M |
| \( B \) | Maximum binding capacity | Moles per unit area of membrane |
| \( K_d \) | Dissociation constant | M |
| \( A \) | Area of cross section | \( \mu \text{m}^2 \) |
| \( D \) | Axial diffusion coefficient | \( \mu \text{m}^2 \text{s}^{-1} \) |
| \( v \) | Transport velocity | \( \mu \text{m s}^{-1} \) |

\[ \frac{1}{1 + \frac{B(z)K_d(z)}{(K_d(z) + c_f)^2}} \]

diffusion,

\[ \frac{1}{A(z)} \frac{\partial}{\partial z} \left( A(z) D(z) \frac{\partial c}{\partial z} \right) \],

and active transport,

\[ \frac{1}{A(z)} \frac{\partial}{\partial z} \left( A(z) v(z) c_f \right) \].

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Figure 9. Contributions of local binding, active transport, and cilium diffusion impediment to OS and synaptic enrichment of PMPs, computed by the DBT model. (A) Schematic of model rod with compartments labeled as in Fig. 2 A. Representative electron micrographs show the geometries of the compartments. Note the many synaptic vesicles filling the spherule (a) and the density and order of OS disc membranes (c). Parameters for each compartment used in all computations are listed: $L$, length; $A_c$, area of cross-section; and $D_{GFP}$, diffusion coefficient of unmodified EGFP. EM images reproduced with permission from a (Schacher et al., 1976), b (Peters et al., 1983), and c (Townes-Anderson et al., 1985). a and b, Scale bars, 1 µm. c, Magnification = 45,000, dia, diameter.

(B–E) DBT predictions of EGFP-GRK1ct18 distribution show that active transport and the connecting cilium diffusion impediment, together, are the major contributors to OS enrichment; local OS binding played a minor role. (F) Predictions of OSEI, $F_{OS}/F_{IS}$ from model traces, and effective $D_{OS}$, found by fitting model FRAPs as described in Fig. 7, plotted versus equivalent binding power. Arrowhead on the $D_{OS}$ line shows that at EBP = 1.5, $D_{OS}$ was approximately twofold lower than that for no binding, as found experimentally for EGFP-GRK1ct18 and EGFP-Far0 versus EGFP (Fig. 7). However, the OSEI was $<$1. The OSEI line shows that tighter OS binding can lead to fivefold (arrowhead) or better OS enrichment, but at the cost of mobility. BP, binding power. (G) Transport velocity versus predicted OSEI, given OS EBP = 1.5. Arrowhead indicates the velocity that produces the observed approximately fivefold OS enrichment, as found experimentally for EGFP-GRK1ct18. (H–J) Higher synapse affinity resulted in significant enrichment (H) and FRAP recovery $t_{1/2}$ $\sim$ 2 min (i and j), similar to experimental results for Far0 (Fig. 4 G), suggesting the distribution of Far0 within the IS is mediated by equilibrium binding alone.
One wonders, however, if the continual turnover of the OSs to daily disc shedding (Besharse et al., 1977; Hollyfield et al., 1977). Depletion of PMPs from the OS, required for super enrichment of the synapse, may operate through a similar, IS-directed continuous recycling mechanism. Our results suggest prenyl-8 probes diffuse through the connecting cilium and into the OS, but are then continuously removed from the ciliary compartment. Recent studies have reached similar conclusions for intrinsic membrane proteins in rods and cells possessing primary cilia (Datta et al., 2015; Ye et al., 2018).

The connecting cilium operates as an active PMP sorting platform
The results suggest the connecting cilium, equivalent to the ciliary transition zone, is an active sorting platform for compartment enrichment. Transport into the OS may operate through an anisotropic diffusion mechanism like that proposed for transport of soluble proteins through the nuclear pore complex (Kee et al., 2012; Jovanovic-Talisman and Zilman, 2017), but imparted by lipid binding chaperone proteins. For example, PrBP-associated GRK1 may pass through the connecting cilium into the OS without encountering the diffusion impediment and then be released by Arl3/Arl13b and the OS localized RP2 (Baehr, 2014; Hilgendorf et al., 2016). Once inside the OS, GRK1 would be in a rapid binding and dissociation cycle with disc membranes, allowing it to equilibrate easily along the OS, but also to diffuse back into the cell body (Fig. 10 B). In the OS to IS direction, a possible mechanism for depleting PMPs from the OS could be removal by the Bardet-Biedl transport complex, the BBSome, which has recently been implicated in removal of membrane proteins from photoreceptor OSs and primary cilia (Datta et al., 2015; Ye et al., 2018). Further study is required to determine how PMPs are sorted at the connecting cilium and ciliary transition zone.

Another mechanism that may drive enrichment of charged proteins across the connecting cilium and within the major rod compartments is voltage gradient–induced electrophoresis. Hagins et al. (1970) showed that the circulating current in dark-adapted rods would produce a significant cytoplasmic potential gradient along the rod axis, with the tip of the OS being positive.
relative to the rod base and IS. This potential difference would produce an electrophoretic driving force on charged molecules, with negatively charged molecules driven toward the OS tip and positively charged molecules driven toward the OS base and into the cell body. The depletion of the NL+8 probe from the OS (Fig. 3 A) suggests electrophoretic enrichment may be an important factor in setting charged protein distributions in rods.

Physiological roles of PMP surface charge and negative disc membrane surface potential

Our results with acylated probes directly demonstrate that disc membranes in live cells bear a significant negative surface charge that impacts PMP dynamics under physiological conditions. This is consistent with previous in vitro results suggesting that the cytoplasmic surface charge of disc membranes, per rhodopsin, is between approximately −5 and −1.5, corresponding to a charge density of approximately (−)0.1 to (−)0.3 per square nanometer (Hubbell, 1990; Tsui et al., 1990; Hubbell et al., 2003). The potential physiological impact of the disc membrane surface charge on transducin disc association was addressed computationally by Kosloff et al. (2008), who showed that Ta has a significantly lower affinity to disc membranes than Taβγ trimer owing to membrane repulsion of negative charges on Ta. Positive surface charge of Tβγ near the farnesyl site was proposed to result in substantially higher disc membrane affinity, enough so that Taβγ trimer has a net disc membrane attraction (Kosloff et al., 2008). It is thought that Ta release from Tβγ may thus drive light-dependent Ta translocation to the IS (reviewed in Calvert et al., 2006) when the number of activated transducins rises above the level of GTPase activating complexes, RG59-Gβ5-R9AP (Lobanova et al., 2007), possibly assisted by association with Unc119 (Zhang et al., 2011). Our results measuring membrane affinity of PMP probes in the OS support this idea. However, they also show that the acylated probe with the weakest membrane affinity (Myr-8) remains OS enriched and that Unc119 association with acylated Tαnt16-EGFP does not result in a distribution significantly different from Myr-8, suggesting additional unknown factors are involved in setting transducin distribution patterns in light- and dark-adapted rods.

The dependence of the affinity of charge-bearing PMPs on membrane surface potential leads to the possibility that binding strength changes depending on the light-adapted state of the photoreceptor. The membrane potential of dark-adapted rods, where a fraction of the OS cyclic nucleotide gated cation channels remain open, is depolarized to approximately −20 mV and the rod OS has a cytosolic Ca2+ concentration of 400–600 nM (McCarthy et al., 1994; Younger et al., 1996). In contrast, rods whose cyclic nucleotide gated channels are completely closed by strong light have a membrane potential of approximately −60 mV and cytosolic Ca2+ of a few nanomolar. Thus, PMPs may undergo a shift in membrane affinity due to changes in the concentrations of charge shielding cations, especially Ca2+, or changes in the protein surface charge, e.g., by phosphorylation. Interestingly, GRK1 was reported to undergo light-dependent phosphorylation (Horner et al., 2005; Osawa et al., 2011), which would reduce its disc membrane affinity and thus reduce probability for activated rhodopsin encounter, possibly explaining the observed reduction in rhodopsin phosphorylation efficiency of phospho-GRK1 (Horner et al., 2005). Our ongoing work on imaging protein dynamics in dark- and light-adapted rods aims to explore these ideas.

Materials and methods

Generation of plasmid constructs and transgenic X. laevis

DNA constructs (Fig. S1) were generated using standard cloning and quickchange mutagenesis methods (Agilent). DNA sequences were placed downstream from the X. laevis opsin promoter to direct expression to rod photoreceptors (Mani et al., 2001). The coding region of each construct was sequenced (Genewiz) and the plasmid was linearized with XhoI endonuclease before restriction enzyme-mediated integration transgenics. Prior to oocyte injection, the plasmid was incubated with XhoI digested X. laevis sperm nuclei. Sperm nuclei were then treated with X. laevis egg extract, and oocytes were fertilized by injection with the sperm nuclei (Kroll and Amaya, 1996; Knox et al., 1998). Embryos were screened for epifluorescence in the eye, and transgenic animals were grown until eyes were large enough for retinal dissection (approximately stage 42 and older).

All procedures and animal handling were performed per the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Sex of animals used in experiments was not determined; thus, equal numbers of males and females were likely used.

Tissue preparation

X. laevis was dark-adapted for 2–12 h before imaging. Retina dissection was done with infrared illumination to minimize rhodopsin activation. Animals were bathed in 0.05% tricaine (ethyl 3-amino-benzoate methanesulfonate; Western Chemical) and decapitated. Eyes were removed and retinas dissected into frog Ringer solution (120 mM NaCl, 2 mM KCl, 10 mM Hepes, 1.6 mM MgCl2, 10 mM glucose, 0.03 mM EDTA, and 1.0 mM CaCl2). Retinas were placed photoreceptor side up and sliced into strips (Najafi et al., 2012). Retinal “chips” were transferred to a custom-made imaging chamber as described previously (Peet et al., 2004; Calvert et al., 2010).

Confocal imaging

Quantitative imaging experiments were performed using a custom-built confocal/multimode microscope described previously (Peet et al., 2004; Calvert et al., 2007, 2010). Briefly, the microscope consists of an inverted stand (Nikon TE2000-U) into which collimated laser beams were introduced via an x-y galvanometer mirror system (Cambridge Technology). Focus was controlled by a piezo objective motor (Physik Instrumente). In confocal mode, E/PAGFP was excited by the 488-nm line of an argon-ion laser (Spectra-Physics 162-C). In two-photon mode, E/PAGFP was excited with a titanium:sapphire laser (Mai Tai HP; Spectra-Physics). The excitation psf was generated by overfilling the back aperture of a 60×/1.2 numerical aperture, water-immersion objective (Plan Apo VC; Nikon) with the expanded, collimated laser beams, which were thus focused to the diffraction limit within the specimen. Fluorescence emission was
detected using avalanche photodiodes (SPCM-AQR-14; PerkinElmer) in the descanned position. Laser intensities were modulated with neutral density wedges (Thorlabs) and/or a Pockels cell (302RM; Conoptics). Data acquisition and instrument control were achieved through a custom LabView system produced in collaboration with Michael Coleman of Coleman Technologies. All imaging was performed at room temperature (18–20°C).

Cells expressing EGFP probes were located via epifluorescence using a GFP cube (96343; Chroma Technology). Cells expressing PAGFP probes were detected via epifluorescence using a violet cube (11005v2; Chroma Technology). 3D confocal scans were performed with sampling frequencies of 0.05–0.26 µm in xy and a z-step of up to 0.2 µm. 3D confocal images of individual cells were obtained by image segmentation and fluorescence distributions were quantified using custom Matlab computer programs. No other image processing that would compromise fluorescence quantification, such as deconvolution, were performed on the image data. 3D volume renderings were generated using VolView (Kitware).

Determination of the psf intensity profile

Direct measurement of the psf profile in our confocal microscope was performed by scanning 0.1 µm diameter fluorescent microspheres (Peet et al., 2004; Calvert et al., 2007; Geneva et al., 2017). Fluorescence profiles of the microspheres were fitted with Gaussians, yielding standard deviations (σ) in xy and in z of σxy = 0.14 µm and σz = 0.56 µm. To estimate the EGFP or PAGFP photoconversion profile or the blurring by convolution, the psf intensity profile was approximated as a 3D Gaussian,

\[ I_{psf}(x, y, z) = a \times \exp \left( -\frac{(x^2 + y^2)}{2\sigma_{xy}^2} \right) \times \exp \left( -\frac{z^2}{2\sigma_z^2} \right), \]

where \( a \) is the intensity maximum at the 3D center of the profile. For this analysis we used a normalized psf profile where \( \int_{xy} \int_{z} I_{psf} = 1 \).

Image and statistical analysis

Image segmentation was performed using custom Matlab programs to isolate individual rod photoreceptors from 3D images of retinal chips. A spline was drawn down the center of the rod photoreceptor, and fluorescence values were collected along this spline. To determine the approximate fluorescence in each compartment without the blurring on the edges of the cells and compartments due to the psf, the 90th to 100th percentile fluorescence intensity values for the synapse, myoid, and ellipsoid regions were averaged and normalized to the total fluorescence of the cell to get the relative concentration value. For cells expressing constructs with lipidation motifs, the nuclei were nearly empty, so the zero to 10th percentile fluorescence values were taken, again to avoid blurring from neighboring, brighter compartments. These values were then averaged. The OS is much larger than the other compartments of the rod, and thus not as susceptible to blurring, so the average of all OS fluorescence values along the spline was used. The value for each compartment in each cell was then corrected for sampling frequency and normalized to total cell fluorescence. Values were then plotted in a box plot in which the red line is the median, and the bottom and top of the box are the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points that aren’t considered outliers. Outliers are any values over the maximum whisker length, which is the length of the interquartile range. Outliers are indicated with a red +. The green asterisk indicates the average. Statistically significant differences and P values were determined with one-way ANOVA and the Tukey–Kramer multiple comparison procedure.

Fluorescence relaxation after photoactivation (FRPa) and fluorescence relaxation after photobleaching (FRPb) experiments

OS diffusion measurements were made by two different methods: FRPa and FRPb. In FRPa experiments, probes encoding PAGFP were expressed in X. laevis rod photoreceptors. Cells expressing the probes were identified by epifluorescence. A rectangular strip in the OS was photoactivated with a Ti:sapphire laser tuned to 820 nm. Fluorescent relaxation throughout the OS was monitored over a time scale of tens of minutes. In FRPb experiments, cells expressing the EGFP version of probes were bleached with a Ti:sapphire laser tuned to 920 nm, either in a rectangular strip in the OS or at the synapse. Fluorescence recovery was monitored over a time scale of tens of minutes.

Diffusion model fitting of OS FRAP experiments

To estimate axial diffusion coefficients of the probes in the ciliary OS, FRAP relaxation curves were fitted with the one-dimensional diffusion model (Theory Eq. A4; cf. Calvert et al. [2010]). FRAP results were normalized to the prebleach fluorescence level (FRPb) or the peak of the photoactivated fluorescence (FRPa). The model was then solved over a range of DOS values and the results fitted to the data by root mean square error minimization (Calvert et al., 2007, 2010). Each cell was modeled individually to take into account variable OS length and variable bleaching or photoactivation patterns.

Immunoprecipitation and Western blotting

A GFP-trap kit (Chromotek) was used to immunoprecipitate EGFP probes and interacting proteins from transgenic X. laevis retinas. For farnesylated probes, Western blots were probed with PDE6D polyclonal antibody from Novus Biologicals (NBP1-32730) and Living Colors A.v. monoclonal antibody (JL-8; 632381) to recognize PrBP and EGFP, respectively. Several Unc119 antibodies were tested, including an Unc119 antibody that was made and generously provided by F. Haeseleer, University of Washington, Seattle, WA (Haeseleer, 2008), an Unc119 polyclonal antibody purchased from Novus Biologicals (NBP1-81708), and Unc119B polyclonal antibody purchased from Invitrogen (PAS-24504). None of these antibodies detected Unc119 in X. laevis retinas.

Mass spectrometry protein identification

To probe for potential LBC proteins, we performed mass spectrometry protein identification on GFP trap pulldowns.

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Sample processing
Immunoprecipitated samples were digested using either the filter-aided sample preparation (FASP) (Wiśniewski et al., 2009) or the digestion in stage tip (iST) (Wei et al., 2014) procedure. For FASP, a mixture of Tris, pH 8.5, SDS, and DTT was added to the sample to a final concentration of 10 mM, 0.4% (wt/wt), and 10 mM. Disulfides were reduced by heating to 95°C for 5 min, and the samples were cooled to room temperature before being transferred to a 10-kD molecular weight cutoff ultrafiltration vessel (OD010C33; Pall). 200 µl of 8 M urea containing 100 mM Tris, pH 8.5, was added, and the filters were centrifuged to near dryness. Cysteine residues were alkylated with 50 mM iodoacetamide for 25 min in the dark, followed by centrifugation. The retained proteins were further washed with three aliquots of urea + Tris, followed by three washes of 50 mM ammonium bicarbonate. Trypsin was added at a ratio of 1:50 (enzyme: sample) in 40 µl of 50 mM ammonium bicarbonate, and digestion was allowed to proceed overnight at 37°C. The resulting peptides were collected in a clean tube, and the filter was washed using 50 µl of 0.5 M NaCl.

Peptides were desalted using mixed-mode cation exchange (MCX) stage tips (Rappsilber et al., 2003). 200-µl tips were packed with two cores of Empore MCX material (SDB-RPS, 3M, 2241) made using 14-gauge blunt needles. The sorbent was conditioned with acetonitrile (ACN), washed with solvent A (3% ACN in water with 0.1% trifluoroacetic acid [TFA]), up to 10 µg of sample loaded, washed twice with solvent A, washed once with solvent B (65% ACN in water with 0.1% TFA), and eluted using 65% ACN in water containing 5% (vol/vol) of ammonium hydroxide. The desalted peptides were dried in a SpeedVac vacuum concentrator.

When using the iST protocol, stage tips were made by heat sealing the bottom of 200-µl tips, and subsequently packing these with two cores of Empore MCX material. Up to 20 µg of protein was added to each tube, followed by addition of 10 M urea to a final concentration of 2 M. Tris(2-carboxyethyl)phosphine and chloroacetic acid were added to a final concentration of 10 and 40 mM, and the reaction was allowed to proceed for 10 min. An equal volume of digestion buffer was added, containing 10% vol/vol ACN, 100 mM Tris, pH 8.5, and 200 ng of trypsin. The tops of the pipette tips were sealed with parafilm. The samples were digested at 37°C for 3 h in a warm air incubator.

Sample recovery and cleanup was performed by removing the parafilm, cutting off the heat-sealed bottoms, and placing the tips in clean 1.5-ml tubes whose lids had premade holes to accommodate the tips. The samples were acidified using 5% trifluoroacetic acid. After spinning through the sample, the MCX material was washed with 2× 50 µl of 0.2% TFA solution, and 60 µl of 65% ACN with 0.2% TFA. The tips were then placed in clean 1.5-ml tubes and the peptides eluted using 75 µl of 65% ACN with 5% ammonium hydroxide. The peptides were dried in a speed-vac.

Liquid Chromatography–Mass Spectrometry (LC-MS)
Samples were dissolved in water containing 2% ACN and 0.5% formic acid to ~0.25 µg/µL. 2 µL (0.5 µg) was injected onto a pulled tip nano-LC column with 75-µm inner diameter packed to 10 cm with 5 µm C18 particles. The peptides were separated using a 60-min gradient from 3–28% ACN over 60 min, followed by a 7-min ramp to 85% ACN. The column was connected inline with the Orbitrap Lumos via a nanoelectrospray source operating at 2.2 kV. The mass spectrometer was operated in data-dependent top speed mode with a cycle time of 2.5 s. MS2 scans were collected at 60,000 resolution with an automatic gain control (AGC) target of 6.0x105 and maximum injection time of 50 ms. Higher-energy collisional dissociation fragmentation was used followed by MS3 scans in the Orbitrap at 15,000 resolution with AGC target 1.0E4 and 100-ms maximum injection time.

Database search
The MS data were searched using SequestHT in Proteome Discoverer (version 2.2) against the X. laevis proteome from Uniprot, containing 42,878 sequences, concatenated with common laboratory contaminant proteins. Enzyme specificity for trypsin was set to semi-tryptic with up to two missed cleavages. Precursor and product ion mass tolerances were 10 ppm and 0.6 D, respectively. Cysteine carbamidomethylation was set as a fixed modification and methionine oxidation as a variable modification. The output was filtered using the Percolator algorithm with strict false discovery rate set to 0.01.

Online supplemental material
Videos 1 and 2 show 3D images of a rod expressing EGFP-Far+8, rotating in different directions. Images were acquired as described for Fig. 4. Voxel containing the 95th percentile (red) and 50th percentile (green) are shown. 3D rendering were performed in VolView. Video 3 shows a 3D image of another rod expressing EGFP-Far+8. Acquisition and processing were as described in Videos 1 and 2. Tables S1, S2, and S3 show mass spectrometry protein identification results from a gel band at the putative molecular weight of Unc119 and two GFP-trap pulldown experiments. Fig. S1 shows distributions of EGFP with neutral, nonlipidated linkers on the N and C termini of GFP. Fig. S2 shows scatter plots of probe distributions versus expression level.

Appendix
Theory
Here we describe a DBT model that was developed to evaluate the impact of diffusion, local active transport, and local binding on the transport kinetics and the steady-state distribution of molecules in rod photoreceptors. Below we address each process individually and then combine them into a single model.

Diffusion
The general diffusion equation is
\[
\frac{\partial c}{\partial t} = D \nabla^2 c, \tag{A1}
\]
where \( c \) is the concentration of diffusing substance, \( t \) is time, \( D \) is the diffusion coefficient, and \( \nabla^2 \) is the Laplacian operator. The rod photoreceptor may be treated as a cylinder with variable area of cross section. In cylindrical coordinates, the general diffusion equation is
\[
\frac{\partial c}{\partial t} = D_r \left( \frac{\partial^2 c}{\partial r^2} + \frac{1}{r} \frac{\partial c}{\partial r} \right) + D_\theta \frac{\partial^2 c}{\partial \theta^2} + D_z \left( \frac{\partial^2 c}{\partial z^2} \right), \tag{A2}
\]
where \( D_r \), \( D_\theta \), and \( D_z \) are the diffusion coefficients in the indicated directions. We have previously shown that axial diffusion of E/PAGFP is significantly slower than radial and angular diffusion, \( D_r = D_\theta >> D_z \). Thus, the derivatives in \( r \) and \( \theta \) can be neglected, and diffusion and distribution of molecules in the rod may be approximated along one spatial dimension, \( z \). Taking this into consideration, Eq. A2 reduces to
\[
\frac{\partial c}{\partial t} = D_{app} \frac{\partial^2 c}{\partial z^2}, \tag{A3}
\]
where \( D_{app} \) is the apparent axial diffusion coefficient within the cell. The area of cross-section of rods and the local diffusivity of molecules varies as a function of axial position. We previously derived the diffusion equation to account for these variations (Calvert et al., 2010),
\[
\frac{\partial c(z, t)}{\partial t} = \frac{1}{A(z)} \frac{\partial \left[ A(z)D(z)\frac{\partial c}{\partial z} \right]}{\partial z}, \tag{A4}
\]
Local binding
In the case of diffusion with binding, we may write the modified diffusion equation (Eq. A3; cf. Eq. 14.2, Crank [1975]),
\[
\frac{\partial c_f}{\partial t} = D_{app} \frac{\partial^2 c}{\partial x^2} - \frac{\partial c_b}{\partial t}, \tag{A5}
\]
where \( c_f \) is the concentration of free, diffusing molecules, and \( c_b \) is the concentration of bound, nondiffusing molecules.
Eq. A4 thus becomes
\[
\frac{\partial c_f(z, t)}{\partial t} = \frac{1}{A(z)} \frac{\partial \left[ A(z)D(z)\frac{\partial c_f}{\partial z} \right]}{\partial z} - \frac{\partial c_b}{\partial t}, \tag{A6}
\]
or with rearrangement,
\[
\frac{\partial c_f}{\partial t} + \frac{\partial c_b}{\partial t} = \frac{1}{A(z)} \frac{\partial}{\partial z} \left[ A(z)D(z)\frac{\partial c_f}{\partial z} \right]. \tag{A7}
\]
Assuming that binding/unbinding is much faster than diffusion, the bound concentration may be treated as an algebraic function of the free concentration,
\[
c_b = f(c_f).
\]
Substituting Eq. A7 for \( c_b \) in Eq. A6,
\[
\frac{\partial c_f}{\partial t} + \frac{\partial f(c_f)}{\partial t} = \frac{1}{A(z)} \frac{\partial}{\partial z} \left[ A(z)D(z)\frac{\partial c_f}{\partial z} \right] \frac{\partial f(c_f)}{\partial c_f} \frac{\partial c_f}{\partial t} = \frac{1}{A(z)} \frac{\partial}{\partial z} \left[ A(z)D(z)\frac{\partial c_f}{\partial z} \right] \tag{A8}
\]
Eq. A8 represents a general result for a variable area of cross-section, variable diffusivity, diffusion, and binding system.
Together, Eqs. A7 and A8 are a partial differential algebraic equation system.
Next consider binding described by a Langmuir isotherm,
\[
f(c_f) = \frac{B \cdot c_f}{K_d + c_f}, \tag{A9}
\]
where \( K_d = k_{-1}/k_1 \) and \( B \) is binding capacity. Then,
\[
\frac{\partial f(c_f)}{\partial c_f} = \frac{B \cdot K_d}{(K_d + c_f)^2} = R, \tag{A10}
\]
where \( R \) represents the binding reaction. Substitution of Eq. A10 into Eq. A8 yields
\[
\frac{\partial c_f}{\partial t} = \frac{1}{1 + R(z)} \frac{\partial}{\partial z} \left[ A(z)D(z)\frac{\partial c_f}{\partial z} \right], \tag{A11}
\]
Note that in the case of invariant \( D(z) \), Eq. A8 becomes
\[
\frac{\partial c_f}{\partial t} = \frac{D}{1 + R(z)} \frac{\partial}{\partial z} \left[ A(z)D(z)\frac{\partial c_f}{\partial z} \right]. \tag{A12}
\]
Eq. A12 states that the effective diffusion coefficient, \( D_e = \frac{D}{1 + R(z)} \), is inversely proportional to the binding reaction, \( R \). Thus, local binding to immobile sites leads to a reduction in mobility that can be quantified by measuring the impact of functional domains appended to E/PAGFP on estimates of \( D \).
Active transport
Calvert et al. (2010) introduced a local active transport component to the one-dimensional diffusion model (Eq. A4). The approach was to introduce a component that moves molecules within a defined volume region in a defined direction and velocity. A variety of mechanisms could underlie such active transport, including advective fluid flow, motor protein-based transport, and other mechanisms resulting in anisotropic transport. The active transport component may be added to Eq. A11,
\[
\frac{\partial c_f}{\partial t} = \frac{1}{1 + B(z)K_d(z)} \frac{1}{A(z)} \frac{\partial}{\partial z} \left[ A(z)D(z)\frac{\partial c_f}{\partial z} \right] + \frac{1}{A(z)} \frac{\partial}{\partial z} \left[ A(z)\frac{\partial c_f}{\partial z} \right] \tag{A13}
\]
where \( v(z) \) is the transport velocity of unbound molecules.
boundary and initial conditions

Eq. A4, and the systems of Eqs. A7 and A11 or A7 and A13 were solved using the numerical method of lines (MOL; Schiesser, 1991; Schiesser and Griffiths, 2009) where the following boundary and initial conditions were applied:

$$\frac{\partial c_f(z,0,t)}{\partial z} = 0, \quad z = 0, \quad (A14)$$

$$c_f(z,0) = c_0(z). \quad (A15)$$

Eq. A14 specifies no flux boundaries (homogeneous Neumann boundary conditions) at the presynapse (z = 0) and the ciliary OS tip (z = L). Eq. A15 specifies the initial distribution of molecules throughout the rod cell. The MOL is an established general algorithm for hyperbolic-parabolic (convexion-diffusion, equations first order in t) partial differential equations (PDEs) in which the spatial (boundary value) independent variables are replaced with algebraic approximations. The resulting system of initial value ordinary differential equations (ODEs) is then solved numerically with a library ODE integrator. In the present case, the ODE integrator is ode15s from the Matlab library. Additional details about the MOL are available in Schiesser (2013). The Matlab routines are available from the corresponding author upon request.

Eqs. A13, A14, and A15 comprise the DBT model that allows evaluation of the impact of spatially variable diffusion, binding, and active transport on the dynamics and steady-state distributions of proteins along the length of the photoreceptor neuron.

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