A multivesicular body-like organelle mediates stimulus-regulated trafficking of olfactory ciliary transduction proteins

Stimulus transduction in cilia of olfactory sensory neurons is mediated by odorant receptors, Gaolf, adenylate cyclase-3, cyclic nucleotide-gated and chloride ion channels. Mechanisms regulating trafficking and localization of these proteins in the dendrite are unknown. By lectin/immunofluorescence staining and in vivo correlative light-electron microscopy (CLEM), we identify a retinitis pigmentosa-2 (RP2), ESCRT-0 and synaptophysin-containing multi-vesicular organelle that is not part of generic recycling/degradative/exosome pathways. The organelle’s intraluminal vesicles contain the olfactory transduction proteins except for Golf subunits Gγ13 and Gβ1. Instead, Gβ1 colocalizes with RP2 on the organelle’s outer membrane. The organelle accumulates in response to stimulus deprivation, while odor stimuli or adenylate cyclase activation cause outer membrane disintegration, release of intraluminal vesicles, and RP2/Gβ1 translocation to the base of olfactory cilia. Together, these findings reveal the existence of a dendritic organelle that mediates both stimulus-regulated storage of olfactory ciliary transduction proteins and membrane-delimited sorting important for G protein heterotrimerization.
channels are composed of homologous subunits. Retinitis pigmentosa-2 (RP2) is implicated in localizing opsin to the outer segment, and mutation of this protein causes the eye disease X-linked retinitis pigmentosa. RP2 is a GTPase-activating protein for ADP ribosylation factor-like GTase Pase 3 (ARL3), which in turn stimulates the release of lipidated membrane proteins from carrier proteins such as the Hexose-binding protein phosphodiesterase subunit delta (PDE5)26-28. In vitro analyses have shown that ARL3 also releases Gβ1 bound to RP2.

Studies of intracellular OR trafficking have revealed that proteins such as REEP and RTPI/2 promote the plasma membrane localization of ORs in heterologous cell types by regulating their exit from the ER or Golgi29,30. Other studies have identified proteins and mechanisms for ciliary targeting and intraglial transport of G protein subunits, CNGA2 and AC3, as well as for desensitization of transduction by β-arrestin2 and clathrin-mediated internalization of ORs into endosomes23,33,34. However, the pathways responsible for transduction protein storage and transport from the ER/Golgi to the soma to sites of ciliary targeting in the dendritic knob are unknown.

Here, we analyze OSNs in vivo by double/triple immunohistochemistry/lectin staining and correlated light–electron microscopy (CLEM), which combines information about protein localization obtained by confocal fluorescence microscopy with high-resolution cellular ultrastructure data obtained by electron microscopy. We identify a dendritic MVB-like organelle that we have named the multivesicular transducosome (MVT). MVTs carry ORs, Gβ1, Gβ1, CNGA2 and TMEM16B and constitutively express ESCRT-0, synaptophysin and RP2. RP2 is associated with Gβ1 in the MVT’s limiting membrane. Odor stimuli as well as direct stimulation of the transduction pathway with forskolin result in disintegration of the limiting membrane, release of ILVs into the dendroplasm and translocation of RP2/Gβ1 to the plasma membrane and dendritic knob. Taken together, these results reveal a previously unrecognized molecular system that is distinct from prototypical MVB pathways and that, in a stimulus-dependent manner, controls the transport, storage and sorting of proteins that mediate GPCR signaling in neurons.

Results
Several ciliary olfactory transduction proteins colocalize in putative dendritic vesicles
We previously showed that AC3 and OR can colocalize in dendrocytic puncta in OSNs. To investigate whether these putative vesicles contain additional olfactory transduction proteins, we performed double- and triple-immunohistochemistry with antibodies that recognize AC3, ORs, CNGA2, Gaolf, Gβ1 and TMEM16B. The anti-OR antibody we used recognizes two closely related ORs (M71 and M72). The results showed that all six transduction proteins colocalized in a few large distinct puncta in dendrites (arrows in Fig. 1b–e). Unlike the other transduction proteins, Gβ1 was also present in the plasma membrane of apical dendritic segments (arrowhead Fig. 1d). In the soma and the part of the dendrite close to the dendritic origin, smaller puncta that did not contain all transduction proteins were present (arrowheads Fig. 1b, c). Unlike canonical olfactory transduction in cilia, transient receptor potential cation channel C2 (TRPC2)-dependent transduction in response to H2S occurs in the dendritic knob. Notably, double immunohistochemistry for TRPC2/CNGA2 and TRPC2/AC3 showed granular TRPC2 signals that did not colocalize with CNGA2 or AC3 (Supplementary Fig. 1). These results indicated that proteins that mediate stimulus transduction within the ciliary compartment, but not in the dendritic knob, were colocalized in putative large dendritic vesicles.

Identification of an AC3 MVB-like dendritic organelle by CLEM
To investigate the ultrastructure of the multi-transduction protein-containing putative vesicles, we performed CLEM of mouse OE sections. The most commonly used markers for CLEM are recombinant proteins with fluorescence tags that can be detected by transmission electron microscopy (TEM) via immunogold or quantum dot labeling. Because genetically modified mice expressing fluorescence-tagged olfactory transduction proteins are not available, we developed a CLEM protocol using conventional immunohistochemistry to visualize OSNs in vivo, an approach that avoids artifacts due to in vitro culturing, protein modification or overpreservation. We successfully developed a protocol through which we could detect AC3 in ultrathin tissue sections by immunofluorescence. The results showed that AC3 was confined to large spherical MVB-like organelles characterized by a single limiting membrane enclosing small spherical ILVs (Fig. 1f–h). Approximately half of the MVB-like organelles were densely packed with ILVs, while the other half had a clear central region without ILVs (Fig. 1f–h, f–h). Supplementary Figs. 2d, e and 3a–b). The mean diameter of these organelles was 662 ± 16 nm (Fig. 2a, b), which was large relative to the previously reported MVB diameters of 256 nm and 400–500 nm in cortical neurons and other cell types, respectively. The mean diameter of the ILVs inside the organelles was 66 ± 0.5 nm, which is similar to the previously reported size of 50–80 nm (Fig. 2b). The thicknesses of the limiting and ILV membranes were 6.7 ± 0.1 and 7.05 ± 0.06 nm, respectively (Fig. 2b), and the average number of ILVs per MVB-like organelle was 36 ± 12 per section (Fig. 2b). The MVB-like organelles lacked luminal tubules or tubular protrusions, which are associated with sorting cargo, protein recycling and transport toward the plasma membrane or the trans-Golgi network. CLEM and TEM also showed that the soma contained lysosomes and autophagosome-like ultrastructures (Supplementary Fig. 3c). These organelles were restricted to the soma, which was in line with the finding that the lysosomal-associated membrane proteins LAMP1 and LAMP2 were also restricted to the soma, as indicated by immunofluorescence (Supplementary Fig. 4). Immunofluorescence also revealed that the transduction proteins in the soma both did and did not colocalize with LAMP1/2 (Supplementary Fig. 4). These results indicated that the MVB-like organelles in dendrites were not associated with a vesicular degradation pathway.

Lectin staining and CLEM confirm that the large distinctive transduction protein-positive puncta are MVTs
The CLEM protocol we used to correlate AC3 immunofluorescence with the MVB-like organelles was not compatible with a number of different antibodies that recognize other olfactory transduction proteins. However, since AC3, M71/72, CNGA2, Gaolf and Gβ1 and CNGA2, TMEM16B, TMEM16B, Gβ1 and TMEM16B were colocalized in dendrites, it is highly likely that the dendritic AC3 MVB-like organelles identified by CLEM also contained multiple transduction proteins. To confirm this assumption, we performed CLEM using two different biotinylated lectins: Dolichos biflorus agglutinin (DBA) and Wisteria floribunda (WFA). By using biotinylated lectins and fluorescence-tagged streptavidin, it is possible to visualize carbohydrate structures in glycoproteins with high efficiency. The rationale for using these lectins is that both are known to exhibit the same distribution as ORs in OE and OR-specific axonal projections to target neurons in the olfactory bulb of a small number (5–10) of OR-specific OSNs, which are distinct from M71/72 expressing ORs. Thus, these lectins can be used as markers of ORs. The similarity between DBA staining and OR staining was further verified by our finding that DBA immunofluorescence in cilia was reduced in response to inhibition of hedgehog signaling in the same way we previously observed for OR immunofluorescence (Supplementary Fig. 5). Collectively, these results indicated that DBA and WFA recognize carbohydrates that are associated with a select group of ORs, which is in line with the fact that N-terminal glycosylation has been shown to be critical for membrane trafficking of ORs. Importantly, similar to ORs, we found that both DBA and WFA colocalized with AC3 and CNGA in dendrites and cilia.

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The exact targets of DBA and WFA are unknown, the results verified that the transduction protein-containing putative vesicles that we

identified by conventional confocal microscopy were MVB-like organelles. Given that these organelles carried several different transduction proteins, we named them MVTs.

Each M71/72-expressing OSN contains a few MVTs in the proximal half of the dendrite

Because each OSN expressing the OR M71 or M72 is surrounded by OSNs expressing other ORs, we were able to examine the number and distribution of MVTs in individual OSNs. The results showed that the majority of M71/72-positive OSNs (>90%) contained one or up to three distinctive MVTs that were restricted to a defined region located approximately in the middle of the dendrite, i.e., at an average position located approximately 40% of the dendrite length distal to the soma (Fig. 3a, b). The majority of OSNs also contained a variable number of smaller dendrites (Fig. 3a, c, d).

Identification of RP2 in the limiting membrane of MVTs

To characterize the MVTs in dendrites in more detail, we performed systematic immunohistochemical analyses with antibodies that recognize proteins (i) that are known to label MVBs, (ii) that regulate vesicle transport and iii) that are implicated in trafficking of glycoproteins or ORs. The following antibodies were used: CD9, PDEδ, β-arrestin2, RAB7, RTP, AP1G1, Clathrin, LMAN2L, COP8 (SEC23), SEC31, GRASP55, GRASP65, RAB10 and RAB35, IFT20 and IFT88. All antibodies labeled organelles or cellular structures unrelated to MVTs (Supplementary Fig. 1). We previously showed that dendritic ORs do not colocalize with RAB4A, RAB6B, RAB8A/B, RAB11A/B or the Golgi markers GM130 and TGN46. Together these results strengthened our notion that the MVT was distinct from prototypic MVBs that form parts of lysosomal/recycling/exocytic pathways.

Immunohistochemical analysis of the MVTs identified RP2 as an MVT-associated protein. Immunofluorescence analyses revealed a circular pattern of RP2 immunofluorescence enclosing the MVTs (arrows in Fig. 4a–c, g, h). This pattern of RP2 immunofluorescence was not observed in the distal dendritic segment (white arrowheads in Fig. 4a, c). Interestingly, the disappearance of RP2 membrane localization correlated spatially with the appearance of RP2 fluorescence in the plasma membrane, with the RP2 fluorescence gradually increasing in intensity toward the dendritic knob (Fig. 4a–e). This change in the subcellular localization of RP2 could be a consequence of RP2 translocation from the limiting membrane to the plasma membrane, in conjunction with disintegration of the limiting membrane of the MVT.
In agreement with this hypothesis, CLEM showed that distal dendrites contained ILV clusters devoid of a limiting membrane (Fig. 4f, Supplementary Fig. 6a, b). By definition, loss of the limiting membrane meant that these structures could not be called MVTs. Furthermore, we could not call the released cargo-containing vesicles ILVs. For clarity, we use the term “ILV” for small transduction protein-containing vesicles located in the dendroplasm. The ILVs’ could appear in clusters that spread out from the electron-lucent luminal space of former MVTs (Supplementary Fig. 6a). Taken together, these results indicated that MVTs “burst” and released ILVs at approximately the middle of dendrites.

ESCRT-0, but not other ESCRT complexes, localizes to the limiting membrane of MVTs

Sorting of cargo and ILVs during the formation of prototypical MVBs is regulated by ESCRT complexes49. Interestingly, we found that MVTs were positive for HGS and STAM1, which form the ESCRT-0 complex that initiates the sequential assembly of MVBs (Fig. 5a, b). The ILV* clusters released by MVTs in the distal dendritic segment did not contain ESCRT-0 proteins (Fig. 5c, d). Thus, ESCRT-0 localized together with RP2 in the limiting membrane of MVTs. However, while RP2 may have relocated upon membrane disintegration, ESCRT-0 was not enriched in the distal dendritic plasma membrane or dendritic knob (Fig. 5c, d). Further analysis showed that proteins of the ESCRT machinery downstream of ESCRT-0, such as ESCRT-I (TSG101) and ESCRT-III (CHMP1A and CHMP4B), were not localized to MVTs (white arrows in Fig. 5e–h). Moreover, ESCRT-IV (VPS4A) was not localized to MVTs but was highly enriched in the dendritic knob (asterisk in Fig. 5h).

Evidence for the initiation of MVT biogenesis in the soma

CLEM provided evidence that biogenesis of MVTs, such as MVTs and clusters of putative future ILVs that were not enclosed by a limiting membrane and were juxtaposed to the Golgi/ER, occurred in the soma (Supplementary Fig. 6c, d). Consistent with the presence of nascent MVTs in the soma, we observed transduction proteins both colocalized and not colocalized with RP2 (Fig. 4g, h). Conversely, we also identified a ring-shaped RP2 immunofluorescence signal without a transduction protein-positive core (green arrowhead in Fig. 4g). We speculated that these RP2-positive vesicles were nascent MVTs that did not contain all types of transduction proteins and therefore were only positive for RP2. This conclusion was supported by results of triple and double immuno/lectin staining as well as CLEM analyses, which indicated that vesicles not containing all transduction proteins were present in the soma and the dendritic origin (Figs. 1b, c, 2c, d and f’’’). As in dendrites, ESCRT-0 colocalized with M71/72 in the soma (Fig. 5a, b). However, unlike in dendrites, small M71/72-containing puncta that colocalized with ESCRT-III were also found in the soma (yellow arrows in Fig. 5f, g). In addition, the soma contained small M71/72 puncta that did not colocalize with ESCRT-0, ESCRT-III or ESCRT-IV and small ESCRT-0’, ESCRT-III’ and ESCRT-IV’ puncta that did not colocalize with
M71/72 (green and red arrowheads in Fig. 5f–h). These results indicated that ESCRT-dependent biogenesis of MVBs, possibly including also MVTs, occurred in the soma. Taken together, these results were in line with the hypothesis that vesicles containing a limited number of different olfactory transduction were generated in the soma and that subsequent homotypic fusion of these vesicles gave rise to the MVT. The extent to which ESCRT proteins regulate this process remains to be determined.

Localization of RP2-binding proteins and synaptophysin
To obtain evidence for a possible causative relationship between known RP2 functions and MVT assembly and disintegration, we analyzed the expression of known RP2 binding partners. RP2 binds to ARL3 between the inner and outer segments of photoreceptors and regulates the unloading of cargo proteins such as opsin and G proteins to the outer segment\(^{26-28,50}\). We found that in OSNs, ARL3 was not localized to and was not in close proximity to MVTs (Supplementary Fig. 7a). Instead, RP2 and ARL3 immunofluorescence overlapped at the ciliary base in the dendritic knob, where the ARL3 effector PDE\(δ\) was also found (Supplementary Fig. 7a, c).

Another RP2-binding protein in the inner segment of photoreceptors is NSF, which is an AAA ATPase that plays a role in the regulation of synaptic vesicle fusion with the presynaptic plasma membrane\(^{51,52}\). NSF immunofluorescence showed a dotted pattern.
in the dendrites (Supplementary Fig. 7d). Even though NSF immunofluorescence was not restricted to MVTs, there was partial overlap, suggesting that NSF could potentially interact with RP2 in the limiting membrane. Synaptophysin is a protein that, like NSF, participates in the fusion of internal membranes by regulating soluble NSF attachment receptor (SNARE) proteins.

Interestingly, synaptophysin was also localized to MVTs (Supplementary Fig. 7e).

Evidence that Golf subunits are not located together in dendrites

Schwarz et al. showed that RP2 binds specifically to Gβ1 in photoreceptors and that this interaction most likely facilitates membrane association and trafficking of Gβ1 prior to Gβ1:Gγ1 heterodimer formation. Double immunohistochemical staining for RP2 and Gβ1 and line scans of immunofluorescence intensity showed that RP2 and Gβ1 fluorescence overlapped precisely in the limiting membrane as well as along the plasma membrane (Fig. 6a, b). RP2 and Gβ1 were also colocalized at the ciliary base in the dendritic knob (asterisk Fig. 6a). This result indicated that RP2 might regulate membrane association and trafficking of Gβ1 not only in photoreceptors but also in OSNs. Since RP2 has been shown to regulate the assembly of the Gβ1:Gγ1 heterodimer in photoreceptors, we analyzed Gγ1 expression. Interestingly, Gγ1 was not localized in MVTs or in the dendritic plasma membrane (arrows in Fig. 6c). Instead, Gγ1 immunofluorescence was observed in the dendritic knob and cilia as well as in dendrosomatic puncta that were unrelated to MVTs.

Release of ILVs from MVTs is olfactory stimulus dependent

The results indicated that ILVs were released from MVTs in the middle of dendrites as a consequence of disintegration of the limiting membrane. To address whether the release of ILVs is regulated by activation of OSNs by odor stimuli, we analyzed unilateral naris occluded mice. Naris occlusion results in sensory deprivation of OSNs ipsilateral to the occluded naris, while OSNs on the non-occluded side receive twice the volume of inhaled air with odors. S100A5 immunohistochemistry was used to confirm successful naris occlusion, as S100A5 is transiently expressed in response to odor stimuli (Fig. 7a). First, the number of MVTs (i.e., double RP2/CNGA2 puncta) in the OE of both nasal cavities was quantified in 12 days old mice that had been subjected to unilateral naris occlusion from postnatal day 5 (Fig. 7b, c). Sensory deprivation increased both the number of OSNs with MVTs and the number of OSNs with >3 MVTs (Fig. 7b, c). These results indicated that stimulus deprivation led to an accumulation of MVTs. We next quantified the ratio of inactive (S100A5+) OSNs with MVTs (red arrow in Fig. 7d) to active (S100A5+) OSNs without MVTs (green arrows in Fig. 7d) in OE on the occluded and non-occluded side. The results showed that naris occlusion increased the percentage of inactive OSNs with MVTs and decreased the percentage of active OSNs without MVTs (compare the blue and green sectors of the pie charts in Fig. 7e). The naris occlusion effect was evident not only relative to the non-occluded side, but also to OE in control (unoperated) mice. This indicated that doubling of the odor/air stream on the non-occluded...
MVTs. However, MVTs were present in a few OSNs that showed weak S100A5 signals (the gray sectors of the pie charts in Fig. 7e). These OSNs likely were in an early or late phase of odor-induced S100A5 accumulation at the time of tissue fixation. Notwithstanding, these results were compatible with the hypothesis that odor stimuli promoted the disintegration of the limiting membrane of MVTs in OSNs, resulting in the release of ILVs.

Regulation of MVTs by forskolin-mediated activation of AC3
The naris occlusion experiment, in which S100A5 was used as an activity marker, revealed the long-term (days) effect of ambient odorants on MVTs. To analyze the possible short-term (sec) effect of olfactory transduction on MVT disintegration, we treated the OE with forskolin ex vivo. Similar to odorants, forskolin activates AC3 and the downstream olfactory transduction cascade. Unlike odorants in inhaled air, which activate OSNs in a temporally uncontrolled manner, forskolin simultaneously activates all OSNs. To exclude OSNs that could be activated by both ambient odorants and forskolin, we focused on OSNs that were inactive (S100A5−) in ambient air. Accordingly, the changes in the numbers of the following categories of OSNs were quantified: (i) S100A5+ OSNs containing MVTs (the red curve in Fig. 7f), (ii) S100A5+ OSNs without MVTs (the yellow curve in Fig. 7f) and (iii) S100A5+ OSNs stimulated by ambient odorants (the black curve Fig. 7f). In agreement with the results of the naris occlusion experiment, forskolin treatment decreased the number of OSNs containing MVTs and conversely increased the number of OSNs without MVTs (Fig. 7f). Moreover, the result of this experiment involving “forced” activation of inactive OSNs showed that the disintegration of the limiting membrane occurred rapidly, i.e., within approximately 30 s. Note that the duration of the experiment (4 min) was too short to result in an increase in the number of S100A5+ OSNs, which would have hindered the analysis.

Odorant-evoked MVT disintegration in freely behaving mice. The naris closure and the forskolin experiments indicated that the MVT’s limiting membrane disintegrated in response to stimulation of olfactory signal transduction. To further address this idea we analyzed the number of RP2+ MVTs in odorant-exposed awake and freely behaving mice. The mouse OE is a mosaic of over thousand different OSN subpopulations, each expressing a defined OR that determines which odorants that subpopulation detects. Acetophenone is an odorant agonist for M71/72 ORs. Double immunohistochemical analysis for RP2 and M71/72 showed that exposing mice to acetophenone decreased the number of M71/72 OSNs with MVTs in the dendrite within minutes (Fig. 8). This result indicated that binding of an odorant to an OR evoked disintegration of the MVT’s limiting membrane.
Discussion

By combining in vivo CLEM with double and triple immunofluorescence and lectin staining and confocal microscopy, we show that most olfactory transduction proteins are transported and stored together in dendrites within large MVB-like dendritic organelles that we have named MVTs. MVTs disintegrate in response to odor stimuli and contain ORs, CNGA2, AC3, Gβ1, Goαlf and TMEM16B which are proteins that directly mediate olfactory transduction within the ciliary compartment (the MVT pathway is schematically outlined in Supplementary Fig. 8). In accordance with this selectivity of cargo, we found that MVTs do not harbor TRPC2, which is a transduction channel present in the dendritic knob, but not cilia60. Moreover, we previously showed that MVTs do not colocalize with the olfactory marker protein (OMP), which regulates transduction within the cytoplasm61,64. We find that ciliary “nontransduction” proteins, such as the intraflagellar transport components IFT20 and IFT88, as well as proteins enriched in the dendritic knob (e.g., ARL3 and VPS4A) are not present in MVTs. MVBs in general are associated with constitutive molecules that make up either the organelle structure or function in vesicle budding, ubiquitination, protein sorting or transport along microtubules. Our previous study and the results herein show that MVTs are devoid of the coat proteins clathrin and caveolin as well as RABs and LAMP1/2, which regulate endocytic recycling and/or degradation. We found that LAMP1/2 and lysosomes are located in the soma but not in dendrites or the dendritic knob. Thus, MVTs in the dendrite are segregated from late endocytic compartments in the cytoplasm of the soma. MVTs are negative for exosome proteins such as CD9, RAB11A/B, RAB35 and TSG101, GRASP55 and GRASP65, which are associated with the “compartment for unconventional protein secretion” (CUPS) and secretory/degradative autophagy pathways61,62. Instead, we found that MVTs are associated with synaptophysin and RP2, which are proteins that have not been previously associated with MVB-like organelles. These results show that MVTs are distinct from other types of MVBs, which are involved in conventional recycling, exocytic and endocytotic trafficking routes. Their large size, their association with synaptophysin, and the lack of both LAMP1 and luminal tubules also distinguish MVTs from previously described atypical KIFC2-associated somatodendritic MVB-like organelles as well as a type of late endosome that mediates ciliary targeting of peripherin 2 in photoreceptors13,45.

MVTs are associated with ESCRT-0 (HGS and STAM1), which initiates the sequential assembly of MVBs by recognizing and binding ubiquitinated cargos, which are thereafter passed to downstream ESCRT complexes1. However, the maintenance of ESCRT-0 in the limiting membrane of assembled MVTs in dendrites does not fit with the conventional model of ESCRT-0 function. During the biogenesis of MVBs, ESCRT-0 disassociates from the limiting membrane prior to the phase in which ILVs are pinched off into the lumen of the MVB5. The HGS-STAM1 complex in the limiting membrane of the MVT might therefore persistently bind ubiquitinated proteins in the limiting membrane in a manner independent of its possible role in MVT biogenesis in the somatic cytoplasm.

MVTs can be in close contact with each other, and MVTs in the soma and the dendritic origin do not always contain all transduction proteins. This suggests that large MVTs may be generated by homotypic fusion. However, MVTs are not associated with Rab11, which promotes the formation of giant MVBs and exocytosis by regulating docking and membrane fusion64,65. Instead, we found that MVTs colocalize with synaptophysin and are closely associated with the RP2-binding protein NSF, which participates in the fusion of internal membranes by regulating SNARE proteins3,35.
Most MVTs are localized at the proximal start of a previously uncharacterized compartment in distal dendrites of OSNs. This compartment is characterized by RP2 expression in the plasma membrane that gradually increases toward the dendritic knob, where RP2 immunofluorescence is the most intense. The distal half of the dendrite normally lacks MVTs and instead contains smaller and weaker immunofluorescent puncta that, based on our CLEM results, lack a limiting membrane and thus correspond to clusters of released ILVs*. Our results further suggest that odorant-induced AC3 activation results in disintegration of the limiting membrane and release of ILVs* from the MVT into the cytoplasm of the apical dendritic segment. The RP2-limiting membrane thus disintegrates at a location where the plasma membrane shows intense RP2 immunofluorescence. Such a reciprocal change in immunofluorescence between membranes is not evident for ESCRT-0 or synaptophysin. These results indicate that RP2 selectively translocates to the plasma membrane as a consequence of disintegration of the limiting membrane, although it cannot be excluded that the limiting membrane integrates with the plasma membrane at the same time. While the mechanism by which the transduction signal, such as increased cAMP/Ca2+ levels and/or depolarization, stimulates membrane disintegration and release of ILVs* remains to be identified, our results show that this occurs on a timescale (<30 s) that is too rapid for alterations in gene expression to play a direct role. Unlike acetophenone and forskolin exposure, sensory deprivation by unilateral naris occlusion resulted in both an increased percentage of OSNs containing MVTs and an increased number of MVTs per OSNs. Together, these results indicate that MVTs function as reservoirs that in response to odorant stimuli release, in the limiting membrane. RP2 deficiency in mice results in mis-localization of cone opsin and reduced rhodopsin levels in the outer segment. Studies in cultured cells have indicated that RP2 regulates vesicle transport between the Golgi and primary cilia. RP2 is a GAP for Arl3 that regulates the assembly and trafficking of membrane-associated protein complexes to primary cilia by stimulating the release of lipidated cargo proteins from their carriers, such as PDE6β,7,23,64,66. Both RP2 and PDE6 are present in the dendritic knobs of OSNs, where they may interact with ARl3, which we found to be specifically localized to the ciliary base and proximal cilia.
proteins such as ESCRT-0, RP2, and synaptophysin. The results suggest that MVTs play an important regulatory role in the compartmentalization and sorting of GPCR signaling components in dendrites. These findings provide evidence for the existence of a previously unknown regulatory protein trafficking mechanism in neurons, laying the foundation for future studies on stimulus-regulated dendritic transport, storage and assembly of GPCR signaling components between the Golgi exit and postsynaptic membranes.

Methods

Animals

All animal experiments were approved by the Local Ethics Committee for Animal Research at the Court of Appeal for the upper northern area of Norrland (Umeå, Sweden). The mice were kept in ICVs on a 12 h light/dark cycle at the Umeå Center for Comparative Biology, Umeå. C57Bl/6J mice of both sexes were analyzed. The mice were sacrificed by cervical dislocation followed by immediate decapitation.

Naris occlusion

Unilateral naris occlusion in mice was achieved by cauterization. At postnatal day five, five pups were anesthetized by subcutaneous administration of a cocktail containing 0.25 mg/kg medetomidine, 2.5 mg/kg midazolam, and 0.025 mg/kg fentanyl. The right nostril of each deeply anesthetized pup was cauterized with a mini electrosurgery unit controlled bipolar forceps (Martin, Germany). Immediately after cauterization of the nostril, the mice were given an antidote (cocktail of 1.25 mg/kg atipamezole and 0.6 mg/kg naloxone) subcutaneously. The pups were transferred to their mother’s cage, and formation of a scar that resulted in complete unilateral naris closure was confirmed by applying a drop of water to the operated nostril and by postmortem examination. Naris closure was maintained for 7 days.

Forskolin and vismodegib treatment

A solution of 50 μm forskolin (CAS: 66575-29-9, catalog: 1099, Tocris, Biotechne) was prepared in fresh Ringer’s solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, 1 mM sodium pyruvate; pH 7.4). Mice were decapitated and the head cut in half along the sagittal, interfrontal and internasal sutures. Then, the nostril septum was removed. One half of the head was incubated in forskolin solution or Ringer’s solution at room temperature (25 °C) for 30, 60, 120 and 240 s (i.e. the time points given in Fig. 7f). At the end of each time point the tissue was washed in PBS and fixed in 4% paraformaldehyde in PBS (PFA; VWR Chemicals, 28794.295; w/v in PBS). Time from start of PBS wash to fixation was -10 s. Five 40 mg/kg i.p. doses of vismodegib (CAS: 879085-55-9, catalog: GDC 0449, Selleckchem) was administered with 12 h intervals and en face preparations were generated to analyze immunofluorescence in cilia.

Odorant exposure

Two weeks old mice were exposed to 50 μl 50% acetonaphone (CAS: 98-86-2, catalog: 42163, Sigma-Aldrich) in DMSO (CAS: 67-68-5, catalog: D4540, Sigma-Aldrich) or DMSO essentially as described. In short, the mouse was placed in a 10x10x8 cm odorless plastic box fitted with a lid and a clean 1 x 1 x 0.3 cm 3MM Whatman paper. The mouse was habituated for 5 min, acetonaphone or DMSO was then placed on the Whatman paper and the lid was closed. Mice were sacrificed after 60, 180 or 600 s exposure (time points given in Fig. 5b) and the nasal tissue was fixed 75–90 s later in 4% PFA.

Preparation of nasal tissue for confocal microscopy

Nasal tissue was dissected, fixed for 4 h in 4% PFA, incubated in 30% sucrose (w/v in PBS) overnight and frozen in OCT (Histolab, Gothenburg, Sweden). The OCT blocks were sectioned using an HM 550 Cryostat Microtome (Microm) at a thickness of 14 μm, air-dried, incubated with citrate buffer (10 mM, pH 6.0) for 5 min at 100 °C, washed in...
PBS and incubated in blocking solution (2% FCS + 0.2% Triton X-100 in PBS). The sections were washed in PBS, incubated overnight with primary antibodies in blocking solution, washed and incubated with secondary antibodies diluted in T-PBS (0.1% Tween-20 in PBS). The sections were then stained with Hoechst (0.1µg/ml) and mounted with fluorescence mounting media (Dako, CA, USA). The details of antibodies used in this study are in supplementary table 1. The lectins biotinylated DBA (L300 Sigma, L6533) and WFA (L100, Sigma, L7166) were visualized with Cy3™ Streptavidin (Jackson ImmunoResearch Europe, 016-160-084). For immunohistochemical analyses results from biological replicates (numbers in figure legends) gave similar results. Imaging was performed with a Leica TCS SP8 confocal system equipped with a Leica DMi8 microscope using an HC PL APO CS2 63x/1.40 N.A. objective. LAS X software was used to acquire confocal images with a voxel size of 0.60 µm and a pixel size of either 0.09 × 0.09 or 0.12 × 0.12 µm². Confocal images with a z-axis thickness of ~8 µm were acquired using LAS X software.

Tissue preparation for CLEM
Tissue was dissected and incubated for 30 min in fixative (2% PFA, Fisher Scientific PA0995; 0.2% glutaraldehyde, TAAB Laboratories Equipment G011/2) in 0.1 M phosphate buffer (PB) pH 7.4. The OE from turbinates was incubated for 10 min at 20 °C in a vacuum (20 Hg) using the PELO BioWise™ Pro+ Microwave Processing System. The tissue was then washed three times for 5 min in PBS and one time for 5 min in 1% glycine/PBS (Merck, cat. No. 104204.0100), transferred to a 1 mm thin sheet of 12% gelatin, and incubated for 30 min at 37 °C. The gelatin-embedded tissue was cut into 1 mm² blocks, cryoprotected overnight in 2.3 M sucrose (WVR Chemicals, 27460.294) in 0.1 M PB and frozen in liquid nitrogen. Ultrathin (75 nm) sections were generated at −120 °C with a Leica UC7 ultramicrotome in a cryochamber. The sections were transferred to a drop of a 1:1 mixture of 2% methylcellulose (Sigma, M-7140), 2.3 M sucrose and 0.1 M PB and mounted on TEM grids with a carbon-coated Formvar and celloidin (Sigma, M71/72 OSNs with or without MVTs following acetophenone exposure to a 1 mm thin sheet of 12% gelatin, and incubated for 30 min at 37 °C. The gelatin-embedded tissue was cut into 1 mm² blocks, cryoprotected overnight in 2.3 M sucrose (WVR Chemicals, 27460.294) in 0.1 M PB and frozen in liquid nitrogen. Ultrathin (75 nm) sections were generated at −120 °C with a Leica UC7 ultramicrotome in a cryochamber. The sections were transferred to a drop of a 1:1 mixture of 2% methylcellulose (Sigma, M-7140), 2.3 M sucrose and 0.1 M PB and mounted on TEM grids with a carbon-coated Formvar film (TAAB Laboratories Equipment, F005). The grids were incubated in PBS with the sections facing the PBS at 37 °C for 30 min, rinsed in PBS, incubated in blocking solution (1% FCS, 0.1% gelatin (w/v) in PBS) for 10 min and incubated overnight in blocking solution containing primary antibody or lectin. The grids were washed six times in blocking solution, incubated at room temperature for 1 h in blocking solution containing secondary antibody or streptavidin, counterstained for 5 min in Hoechst/PBS (0.1µg/ml), washed three times with blocking solution, washed four times in Milli-Q® water and mounted on microscope slides in 50 µl of 50% glycerol (Sigma Aldrich, csa No. 56-81-5). The overview and ROIs were scanned using an HC PL APO CS2 20x/0.75 IMM objective and HC PL APO CS2 63x/1.40 N.A objective, respectively. The scanning grids were acquired using LAS X software.

Data that support the findings of this study is present in the paper, supplementary information, Source Data and the Zenodo database (10.5281/zenodo.7194768). Source data are provided with this paper.

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D.K.M. planned and performed experiments, analyzed data and wrote the manuscript. A.B. and S.B. planned the experiments, supervised the study and wrote the manuscript.

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