SIPA1L2 controls trafficking and local signaling of TrkB-containing amphisomes at presynaptic terminals

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Amphisomes are organelles of the autophagy pathway that result from the fusion of autophagosomes with late endosomes. While biogenesis of autophagosomes and late endosomes occurs continuously at axon terminals, non-degradative roles of autophagy at boutons are barely described. Here, we show that in neurons BDNF/TrkB traffic in amphisomes that signal locally at presynaptic boutons during retrograde transport to the soma. This is orchestrated by the Rap GTPase-activating (RapGAP) protein SIPA1L2, which connects TrkB amphisomes to a dynein motor. The autophagosomal protein LC3 regulates RapGAP activity of SIPA1L2 and controls retrograde trafficking and local signaling of TrkB. Following induction of presynaptic plasticity, amphisomes dissociate from dynein at boutons enabling local signaling and promoting transmitter release. Accordingly, sipa1l2 knockout mice show impaired BDNF-dependent presynaptic plasticity. Taken together, the data suggest that in hippocampal neurons, TrkB-signaling endosomes are in fact amphisomes that during retrograde transport have local signaling capacity in the context of presynaptic plasticity.
DNF/TrkB signaling is required for a variety of neuronal functions including neurotransmission and synaptic plasticity. Upon binding to DNF, DNF/TrkB is internalized via pinocytosis, or in a clathrin-dependent manner to vesicular compartments called signaling endosomes that might allow for local signaling. Signaling endosomes are predominantly generated at axon terminals by different mechanisms and constitute long-lived organelles that persist while undergoing transport from nerve terminals to the neuronal soma. In neurons, retrograde trafficking of TrkB signaling endosomes constitutes an important long-range signaling mechanism that conveys information on presynaptic activity to the cell soma. How endosomal sorting is accomplished in these organelles and how they escape from the degradative pathway remains still unclear.

A key consequence of TrkB signaling is sustained ERK activation, a process achieved via activation of the small GTPase Rap1 (Supplementary Fig. 1a). Accordingly, prenylated Rap1 is associated with TrkB signaling endosomes and regulation of its GTPase activity is, therefore, a likely mechanism to control local TrkB signaling.

A regulator of Rap1 is SIPA1L2 (also known as SPAR2), a member of the SIPA1L family of neuronal RapGAPs (Supplementary Fig. 1b–d). The protein is most abundant in granule cells of the dentate gyrus (DG) and cerebellum and shows RapGAP activity for Rap1 and Rap2. This RapGAP activity promotes the intrinsic GTPase activity of Rap1/2 that catalyzes the hydrolysis of GTP to GDP and inactivates Rap1/2 and consequently, ERK signaling. Here, we report that sipa1l2 knockout (ko) mice show impaired long-term potentiation (LTP) at mossy fiber (MF) synapses and spatial pattern separation, which requires MF plasticity. MF-LTP is an NMDA receptor-independent form of LTP that is expressed pre-synaptically and depends upon local DNF/TrkB signaling.

Accordingly, we found that SIPA1L2 directly binds to TrkB and application of a TAT-peptide encompassing the binding region for TrkB in SIPA1L2 induces a similar phenotype in vivo and in vitro in wild-type (wt) mice like those observed in sipa1l2 ko mice. We found that SIPA1L2 links the receptor tyrosine kinase to a dynein motor via a direct interaction with the adaptor protein Snapin which allows retrograde transport. Interestingly, SIPA1L2 concurrently interacts with Light chain 3 (LC3), a marker for autophagosomes that is involved in substrate selection, and this interaction promotes SIPA1L2 RapGAP activity. While autophagosomes are continuously generated at axon terminals, very little is known about the synaptic role of autophagy. Here we show that SIPA1L2 associates with amphisomes, organelles from the autophagic pathway that result from the fusion of autophagosomes with late endosomes, that are positive for the late endosome marker Rab7 as well as LC3 and TrkB. This configuration allows LC3 to tightly control TrkB signaling via interaction with SIPA1L2, which increases the RapGAP activity and promotes Rap1/ERK inactivation. We show that these amphisomes traffic retrogradely along axons, stop at presynaptic boutons and both motility and signaling are controlled by SIPA1L2’s RapGAP activity that reduces the velocity of amphisome transport. Presynaptic LTP induces a protein kinase A (PKA)-dependent dissociation of the SIPA1L2/Snapin complex from dynein intermediate chain (DIC). This increases dwelling time of the amphisome at presynaptic boutons and PKA phosphorylation of SIPA1L2 reduces RapGAP activity, therefore, enabling local TrkB signaling at boutons, which in turn promotes neurotransmitter release.

Collectively, the data suggest that retrograde axonal transport of DNF/TrkB occurs in neuronal amphisomes that allow local control of TrkB signaling and are involved in plasticity-relevant local signaling at presynaptic boutons.

Results

MF-LTP and pattern separation deficits in sipa1l2−/− mice. To study the neuronal function of SIPA1L2, we generated sipa1l2 ko mice (Supplementary Fig. 2a–d). No major morphological abnormalities were observed in the cerebellum and DG (Supplementary Fig. 2e–k), motor learning or coordination (Supplementary Fig. 2l–m). The number of adult-born granule cells and measures of general DG excitability and postsynaptic function were all normal in sipa1l2−/− mice (Supplementary Figs. 2n–o and 3). However, a significant deficit was found when we assessed MF LTP, a form of plasticity presynaptically expressed at MF boutons of DG granule cells (Fig. 1a, b). Accordingly, we also observed a strong impairment in spatial pattern separation, a cognitive process associated with proper DG function and MF LTP12–16, that is responsible for the disambiguation and independent storage of similar memories (Fig. 1c–e). No changes were found in novel object location and recognition paradigms that are sensitive to perturbation of synaptic function in hippocampal CA1 neurons (Fig. 1f). Spatial pattern separation as well as MF LTP have been shown to depend on DNF/TrkB signaling in the DG11,13 and we indeed found a reduction in MF LTP when we chelated endogenous BDNF during perfusion of slices with TrkB-Fc bodies (Fig. 1g, h), resembling the decline in late phase LTP found in sipa1l2−/− mice.

The cytoplasmic domain of TrkB directly binds to SIPA1L2. These experiments raised the question of whether SIPA1L2 might be involved in presynaptic DNF/TrkB signaling. DNF/TrkB are internalized at distal axons and transported retrogradely in a dynein-dependent manner as signaling endosomes associated with Rap14,8. We found SIPA1L2 prominently present at presynaptic terminals of primary neurons labeled by the presynaptic marker Synaptophysin 1 and the axonal marker Tau (Fig. 2a, b). We, therefore, wondered whether SIPA1L2 and TrkB might be part of a single protein complex in vivo. SIPA1L2 showed an overlapping distribution with Rap1 as well as with TrkB in subcellular fractionation experiments (Supplementary Fig. 4a). Coinmunoprecipitation experiments from extracts of rat hippocampi revealed that the protein is indeed present in immunoprecipitates generated with a TrkB-specific antibody andvice versa, TrkB could be precipitated with a SIPA1L2 antibody (Fig. 2c; Supplementary Fig. 4b). Moreover, full-length TrkB and SIPA1L2 are present in transport complexes immunoprecipitated by an antibody directed against DIC (Fig. 2d) and both proteins are localized to axonal terminals in hippocampal primary neurons (Fig. 2e) where they were found in very close association as revealed by STED imaging (Fig. 2f, g).

Next, we mapped the binding region in both proteins with Yeast Two Hybrid (YTH) and found that the ActII-domain of SIPA1L2 interacts in the TrkB cytosolic domain with the first 12 juxtamembrane amino acids (aa) (454–465) and the first 23 aa of the tyrosine kinase domain (537–559) (Supplementary Fig. 4c–d). Bacterially expressed GST- and MBP-fusion proteins revealed a direct interaction of cytoplasmic TrkB with the ActII but not the ActI domain of SIPA1L2 in pull-down assays (Supplementary Fig. 4e). We isolated 14 aa in SIPA1L2-ActI that are crucial for the association with TrkB (Fig. 2h; Supplementary Fig. 4f–h) and that happen to be unique to SIPA1L2 when compared to other SIPA1L family members (Supplementary Fig. 1c) and designed a TAT-peptide containing this region. This TAT-peptide competed for binding to TrkB in a pull-down assay (Fig. 2i) and when infused into the DG of wt animals (Fig. 2j; Supplementary Fig. 4i) induced a deficit in spatial pattern separation (Fig. 2k, l) similar to the one observed in sipa1l2−/− mice (Fig. 1e). In addition, the bath perfusion of the TAT-peptide also reduced the amplitude of MF-LTP in acute
slices (Fig. 2m). Thus, interruption of the Sipa1l2-TrkB interaction in wt mice mimics the deficits of sipa1l2−/− mice.

SIPA1L2 interacts with Snapin and enables TrkB trafficking. Dynein-dependent retrograde trafficking of TrkB-signaling endosomes in axons requires Snapin, an adapter protein that recruits dynein by interacting with DIC15. However, whether Snapin directly or indirectly interacts with its cargo is still unclear. Heterologous coimmunoprecipitation experiments using tag-specific antibodies revealed that Snapin associates with the

endosomes in axons in wt mice mimics the deficits of sipa1l2−/− mice.

Fig. 1 sipa1l2−/− mice exhibit impaired MF plasticity and deficits in pattern separation. a MF-LTP was induced in acute slices using a high-frequency stimulus (HFS) protocol. The NMDA receptor antagonist D-APV (50 μM) was present during baseline recordings and LTP induction (shaded blue). Bath application of the group II mGluR agonist DCG-IV (2 μM) that suppresses synaptic transmission at the MF pathway was performed during the last 10 min (shaded gray) of each experiment to control input specificity. Left, the average values of fEPSP amplitudes upon MF-LTP induction. Right, fEPSP amplitudes during the last 45–70 min following MF-LTP induction. b Averaged fEPSP amplitudes recorded during the last 10 min of a before DCG-IV application (Mann-Whitney U test). c Timeline (upper panel) and schematic representation of the object distribution (lower panel) of the spatial pattern separation test. Gray bars indicate 10-min intervals. During the sample phase (red-shaded) objects (A1–3) in the similar location recognition group (SLR) were placed closer while objects in the dissimilar location recognition group (dSLR) (A1–3) were placed farther away from each other. During choice phase (gray-shaded), a new object (A4) was introduced. Animals from the SLR find A4 closer to positions A2–A3 and have a higher demand for pattern separation than those from the dSLR. Filled circles (A1–4) represent object location. Open circles indicate the absence of objects. d Exploration time of sipa1l2 wt and ko animals in A1–3 during the sample phase (two-way ANOVA). e Discrimination index during choice phase in the SLR and dSLR groups (unpaired Student’s t test). f Discrimination index of sipa1l2 wt and ko animals during the novel object location recognition and object recognition test (unpaired Student’s t test). g, h Left, average fEPSP amplitudes upon MF-LTP induction as explained in a in control and BDNF-depleted slices (TrkB-Fc, 5 μg/mL). Right, averaged fEPSP amplitudes obtained during the last 10 min of g prior DCG-IV application (Mann-Whitney U test). Bars and error bars depict mean ± SEM in all graphs. Circles represent mean values from individual subjects (d-f) or slices (a, b, g, h). n.s. not significant. "*" indicates P ≤ 0.05; "***" indicates P ≤ 0.001.
**Fig. 2** SIPA1L2/TrkB interaction at presynapses is crucial for the function of the DG. 

**a** Confocal images of rat hippocampal neurons immunostained against SIPA1L2, Synaptophysin 1 and Tau. Scale bar is 5 μm.

**b** Pearson’s correlation coefficient calculated for SIPA1L2 and Synaptophysin 1 from a. Circles represent averaged values per region of interest (ROI) analyzed from three independent images.

**c** Endogenous TrkB coimmunoprecipitates SIPA1L2 from rat hippocampal lysates. The two bands in TrkB correspond to the full length (TrkB-FL) and the truncated form (TrkBt). Goat anti-TrkB antibody (R&D) is used for precipitation and detection.

**d** DIC coimmunoprecipitates SIPA1L2 and TrkB but not GM130 from mouse whole brain crude light membrane fraction. Note that the band revealing TrkB corresponds to the full-length form.

**e** Confocal images from hippocampal neurons immunostained against SIPA1L2, TrkB and Synaptophysin 1 (confocal). Line profiles (g) indicate relative intensities for STED channels along 1 μm.

**f** STED images from hippocampal neurons immunostained against SIPA1L2, TrkB and Synaptophysin 1 (confocal). Line profiles (g) indicate relative intensities for STED channels along 1 μm.

**g** Pulse-chase experiment for motif identification (GST pulldown assay) performed in the presence of TrkB.

**h** Exploring time from mice injected with TAT-SIPA1L2 or TAT-scr during the sample phase (two-way ANOVA). Discrimination indexes obtained during the choice phase in SLR and dSLR groups injected with either TAT-SIPA1L2 or TAT-scr.

**i** Pull-down assay between TrkB and ActI-SIPA1L2 in the presence of 10× TAT binding peptide (TAT) or 10× scrambled peptide (TAT-scr).

**j** Cartoon representing the timeline used for the pattern separation test performed in wt animals infused with TAT peptides and the location of the infusion (red dot).

**k** Exploring time from mice injected with TAT-SIPA1L2 or TAT-scr during the sample phase (two-way ANOVA). Discrimination indexes obtained during the choice phase in SLR and dSLR groups injected with either TAT-SIPA1L2 or TAT-scr.

**l** The number of subjects is depicted in k (unpaired Student’s t test).

**m** fEPSP amplitudes recorded during the last 45–70 min after MF-LTP upon bath perfusion of TAT-SIPA1L2 or TAT-scr peptides. Right, averaged values of fEPSP amplitudes during last 10 min of LTP recording before DCG IV application (Mann-Whitney U test). Bars and error bars represent data as mean ± SEM in all graphs. Circles represent mean values of individual subjects (k-l) or slices (m). *** indicates P < 0.05; **** indicates P ≤ 0.0001.
central region of SIPA1L2 encompassing the RapGAP and PDZ domains (Fig. 3a; Supplementary Fig. 5a). The tagged RapGAP-PDZ domain corecruits GF1-Snapin (Fig. 3b, c) and both proteins cotraffic in COS7 cells (Fig. 3d, e). Importantly, Snapin, TrkB and SIPA1L2 colocalize in axons of primary neurons (Fig. 3f) and live-imaging experiments using fluorescence-tagged proteins revealed a high percentage of cotrajectories in axons whose motility was predominantly retrograde (Fig. 3g–k; Supplementary Fig. 5b). Analysis of single TrkB and SIPA1L2 trajectories showed similar motility profiles for both proteins individually, in agreement with their cotrafficking (Fig. 3b). Of note, SIPA1L2 imaged in dendrites remained immobile (Supplementary Fig. 5c–d). We further confirmed this by shRNA-based knockdown (KD) of Snapin which reduced the motility of TrkB and SIPA1L2 and resulted in largely stationary complexes at the expense of less retrograde movement (Fig. 3l, m; Supplementary Movies 1–2). Further, TrkB was found to remain mainly stationary in sipa1l2−/− neurons, supporting the role of SIPA1L2 in retrograde TrkB trafficking by mediating attachment to dynein via Snapin (Fig. 3n, o).

LC3b direct association to SIPA1L2 modulates RapGAP activity. Autophagosomes are continuously generated at distal axons and are proposed to acquire Snapin and the dynein motor complex from late endosomes. The fusion of autophagosomes with late endosomes generates amphisomes and facilitates the retrograde trafficking of autophagosomes19. In MRC5 cells we observed a prominent colocalization of SIPA1L2 with the autophagosomal marker LC3b but not with markers for lysosomes (LAMP1) and multivesicular bodies (CHMP4B) (Supplementary Fig. 5e–f). Immunostaining of SIPA1L2, TrkB and LC3 in primary hippocampal neurons revealed extensive colocalization of all three proteins in axons (Supplementary Fig. 5g) that was further enhanced by incubation with BDNF as quantified by Manders’ coefficient calculated for protein pairs (Supplementary Fig. 5h–i). In addition, live imaging revealed largely retrograde cotrafficking of TrkB-GFP and tRFP-LC3b in a SIPA1L2-dependent manner (see above, Fig. 3n, o).

Interestingly, the RapGAP domain of SIPA1L2 contains a LIR-motif (FxxL-motif) for LC3-binding that is highly conserved in mammals and among all SIPA1L family members (ref. 20; Supplementary Fig. 1c). In endogenous coimmunoprecipitation experiments performed with an anti-DIC antibody to isolate transported complexes, we found that LC3c coprecipitates with SIPA1L2 and TrkB (Fig. 2d). In addition, heterologous coimmunoprecipitation experiments conducted with tag-specific antibodies revealed an interaction of the RapGAP domain with endogenous LC3 in HEK293T cell lysates (Fig. 4a; Supplementary Fig. 5a). Binding to LC3c was weaker when we expressed SIPA1L2 carrying point mutations within the LIR motif (Fig. 4b). Moreover, the RapGAP domain fused to GST efficiently pulled down endogenous LC3 from a rat brain lysate (Fig. 4c). The catalytically active RapGAP domain of SIPA1L2 (ref. 21; aa 470–838; Supplementary Figs. 1d; 5j–k) also showed a direct interaction with bacterially expressed His-LC3b in a pull-down assay (Fig. 4d) and this interaction was not affected by the inclusion of Snapin in the pull-down buffer (Fig. 4e). Reciprocally, the association with Snapin was not reduced by mutation of the LIR motif (Fig. 4f). Thus, it seems that both Snapin and LC3b concomitantly bind to SIPA1L2 and that the presence of Snapin does not prevent the binding of SIPA1L2 to LC3b.

In the next set of experiments, we determined whether the binding of LC3b might modulate RapGAP activity. To this end, we extracted SIPA1L2 expressed in HEK293T cells and pulled down endogenous GFP-bound Rap1 with RalGDS-RBD coupled to GST-matrix. As a negative control, we used an SIPA1L2 mutant carrying a point mutation within the Asn-thumb (SIPA1L2-N705A) that renders the RapGAP domain inactive (Fig. 4g, h; Supplementary Fig. 1c). RapGAP activity of SIPA1L2 was abolished when we expressed the SIPA1L2 LIR-mutant (Fig. 4i, j). On the contrary, RapGAP activity was enhanced in the presence of recombinant His-LC3b (Fig. 4k–m). Collectively, these data suggest that the interaction of LC3b with the RapGAP domain modulates the catalytic activity of SIPA1L2.

SIPA1L2 controls retrograde trafficking of TrkB/LC3b. Rap signaling is necessary for sustained activation of ERK22–24 which in turn has been shown to recruit Dynein and to promote retrograde trafficking of TrkB25. Therefore, we asked whether the RapGAP activity of SIPA1L2 might modulate trafficking of the complex. Live-imaging of rat hippocampal neurons confirmed cotrafficking of LC3b and SIPA1L2 in a predominantly retrograde direction (Fig. 5a–c). LC3b trajectories were in the majority of cases positive for SIPA1L2 (Fig. 5d). A SIPA1L2-RapGAP dead mutant that is incapable of terminating Rap-signaling also showed cotrajectories with LC3b (Fig. 5c, d). However, under these conditions, the instant velocity and the run-length of the SIPA1L2-LC3b complexes were significantly enhanced (Fig. 5b, e–g), in agreement with an increased ERK activation and recruitment of Dynein25. Because of the effect of the RapGAP activity on the run-length of the complex, we hypothesized that the SIPA1L2/LC3b stops might occur at presynaptic boutons and that this might be controlled by the RapGAP activity of SIPA1L2. Live-imaging of short axonal segments (30–40 µm) where active presynaptic terminals were identified using Synaptotagmin1Oy-ster650 live-antibody labeling demonstrated that SIPA1L2/LC3b complexes preferentially stopped at single, active boutons (Fig. 5a, b, h), while no changes in the number of visited boutons per axonal segment between WT- and RapGAP-dead SIPA1L2/LC3b trajectories was observed (Fig. 5i), we found that the lack of RapGAP activity strongly diminished the dwelling times of SIPA1L2/LC3b at presynaptic boutons (Fig. 5j, k). Taken together, these data indicate that the RapGAP activity of SIPA1L2 controls the trafficking of the complex which retrogradely trafficks stopping at synaptic boutons.

cLTP prolongs the stopovers of TrkB-amphisomes at boutons. Presynaptic LTP results in activation of PKA at MF boutons and enhanced synaptic function that is largely mediated by PKA-dependent phosphorylation of different components of the presynaptic release machinery.26–30. PKA-mediated phosphorylation of Snapsin at Ser50 is crucial for its dissociation from the DIC and a phosphomimetic Snapsin-S50D mutant is largely immobile31. Heterologous coimmunoprecipitation experiments revealed that both phosphomimetic (S50D) as well as phospho-deficient (S50A) Snapsin interact with tRFP tagged SIPA1L2-470-1025 (Fig. 6a). However, in contrast to the phospho-deficient protein, DIC did not communoprecipitate with phosphomimetic Snapsin (Fig. 6a), indicating that PKA-dependent phosphorylation induces the dissociation of the protein from DIC. Consistent with previous work31, time-lapse imaging revealed that phosphomimetic Snapsin is largely stationary and was often found in proximity to immobile GFP-SIPA1L2-mCherry at axon terminals (Fig. 6b, c). We therefore next wondered whether changes in synaptic activity could affect amphisome trafficking among presynaptic terminals. Trafficking of SIPA1L2/LC3b among presynaptic terminals labeled by anti-Synaptotagmin1Oy-ster650 antibodies revealed an enhancement in dwelling time upon induction of chemical LTP (cLTP) (Fig. 6d–f). The PKA inhibitor H89 prevented this effect (Fig. 6e, f).
SIPA1L2 associates and cotrajectories is represented in shRNA-based Snapin KD or Scr control together with anterograde. Scale bars are 10 μm.

Relative motility of TrkB and SIPA1L2 as a percentage of total trajectories (TrkB i +/–). Total number of cotrajectories is 60.

GFP-Snapin coimmunoprecipitates RapGAP- and PDZ-SIPA1L2-tRFP from HEK293T cells extracts. The upper blot shows the signal from the anti-tagRFP and anti-GFP antibodies. Green arrow indicates immunoprecipitated GFP-Snapin. The lower blot represents the input. Note that the anti-trRFP antibody used also recognizes GFP.

Representative images from rat hippocampal neurons stained against TrkB, SIPA1L2, Snapin, and Tau.

Representative kymographs from neurons overexpressing TrkB-GFP and fl-SIPA1L2-mCherry. Relative motility of SIPA1L2/TrkB cotrajectories is indicated in h. Total number of cotrajectories is 84. I Relative motility of TrkB and SIPA1L2 as a percentage of total trajectories (TrkB = 284 trajectories; SIPA1L2 = 146; the number of axons is depicted in h). j, k Representative kymographs from neurons overexpressing GFP-Snapin and fl-SIPA1L2-mCherry. Relative motility of SIPA1L2/Snipcotrajectories is represented in k. Total number of cotrajectories is 60. l, m Representative kymographs from neurons overexpressing either shRNA-based Snapin KD or Scr control together with fl-SIPA1L2-mCherry and TrkB-SNAP (+SiR647). GFP represents transfection control. Relative motility of SIPA1L2/TrkB cotrajectories is depicted in m. N = analyzed axons (nonparametric Kolmogorov-Smirnov test). n, o Representative kymographs (g, j, l, n) were generated from axons of rat hippocampal neurons. Relative motility (h, k, m, o) is shown as percentage of total cotrajectories. Bars and error bars represent data as mean ± SEM. N number in the graph corresponds to the number of axons. Symbols in columns represent values from single axons. Arrows indicate cotrajectories. R retrograde, S stationary, A anterograde. Scale bars are 10 μm (b) and 5 μm (b, inset; d-f). *** indicates P ≤ 0.01; **** indicates P ≤ 0.001.

Fig. 3 SIPA1L2 associates and cotrajectories with Snapin and TrkB. a GFP-Snapin coimmunoprecipitates RapGAP- and PDZ-SIPA1L2-tRFP from HEK293T cells extracts. The upper blot shows the signal from the anti-tagRFP and anti-GFP antibodies. Green arrow indicates immunoprecipitated GFP-Snapin. The lower blot represents the input. Note that the anti-trRFP antibody used also recognizes GFP. b, c Representative images (b) from the recruitment assay performed in COS7 cells using SIPA1L2-470-1025-tRFP or tRFP as control and GFP-Snapin. Insets are shown in the lower panel. The line profile (c) was generated from the line in the inset. d, e Snap-shots of fl-SIPA1L2-mCherry and GFP-Snapin cotrajectories in COS7 cells (d) and corresponding kymograph (e). Orange line in d represents the trajectory used for kymograph generation (e). f Confocal images from rat hippocampal neurons stained against TrkB, SIPA1L2, Snapin, and Tau. g, h Representative kymographs from neurons overexpressing TrkB-GFP and fl-SIPA1L2-mCherry. Relative motility of SIPA1L2/TrkB cotrajectories is indicated in h. Total number of cotrajectories is 84. i Relative motility of TrkB and SIPA1L2 as a percentage of total trajectories (TrkB = 284 trajectories; SIPA1L2 = 146; the number of axons is depicted in h). j, k Representative kymographs from neurons overexpressing GFP-Snapin and fl-SIPA1L2-mCherry. Relative motility of SIPA1L2/Snipcotrajectories is represented in k. Total number of cotrajectories is 60. l, m Representative kymographs from neurons overexpressing either shRNA-based Snapin KD or Scr control together with fl-SIPA1L2-mCherry and TrkB-SNAP (+SiR647). GFP represents transfection control. Relative motility of SIPA1L2/TrkB cotrajectories is depicted in m. N = analyzed axons (nonparametric Kolmogorov-Smirnov test). n, o Representative kymographs (g, j, l, n) were generated from axons of rat hippocampal neurons. Relative motility (h, k, m, o) is shown as percentage of total cotrajectories. Bars and error bars represent data as mean ± SEM. N number in the graph corresponds to the number of axons. Symbols in columns represent values from single axons. Arrows indicate cotrajectories. R retrograde, S stationary, A anterograde. Scale bars are 10 μm (b) and 5 μm (b, inset; d-f). *** indicates P ≤ 0.01; **** indicates P ≤ 0.001.
It has been reported that PKA-dependent phosphorylation of Ser499 of Rap1GAP negatively regulates RapGAP activity. Sequence analysis of SIPA1L2 revealed a high scoring PKA phosphorylation motif (S990-RxxpS motif/0.757; Supplementary Fig. 1d) that is located close to the region that corresponds to the regulatory PKA sites (pSer 499) of Rap1GAP and that is well preserved between SIPA1L2 family members (Supplementary Fig. 1c). When we tested the hypothesis that PKA in analogy to Rap1GAP might negatively regulate RapGAP activity of SIPA1L2, we found that phosphomimetic SIPA1L2-470-1025-S990D indeed hydrolyzed very little recombinant Rap1b-GTP (Fig. 6g±i), indicating a negative regulation of RapGAP activity by PKA. Collectively these data suggest that following induction of presynaptic plasticity, PKA phosphorylation of Snapin induces the dissociation of the amphisome from Dynemin and enhances its residing time at presynaptic boutons. Concomitant phosphorylation of SIPA1L2 diminishes its RapGAP activity and thereby potentially facilitates ERK signaling. 

Amphisomes activate ERK at boutons and enhance release. Amphisomes are believed to be transient intermediate organelles that in nonneuronal cells rapidly enter a degradative lysosomal

Fig. 4 LC3 interacts with the RapGAP domain of SIPA1L2 and promotes RapGAP activity. a RapGAP-GFP but not GFP immunoprecipitates endogenous LC3 from a HEK293T cell extract. b Heterologous Co-IP from HEK293T cells showing a reduced interaction between GFP-LC3b and SIPA1L2-470-838, which harbors a mutation within the LIR motif (FxxL/AxxA), when compared to fl-SIPA1L2-mCherry, which is well co-immunoprecipitated with recombinant Rap1b-GTP (Fig. 6g±i). c When we tested the hypothesis that PKA in analogy to Rap1GAP might negatively regulate RapGAP activity of SIPA1L2, we found that phosphomimetic SIPA1L2-470-1025-S990D indeed hydrolyzed very little recombinant Rap1b-GTP (Fig. 6g±i), indicating a negative regulation of RapGAP activity by PKA. Collectively these data suggest that following induction of presynaptic plasticity, PKA phosphorylation of Snapin induces the dissociation of the amphisome from Dynemin and enhances its residing time at presynaptic boutons. Concomitant phosphorylation of SIPA1L2 diminishes its RapGAP activity and thereby potentially facilitates ERK signaling. 

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It has been reported that PKA-dependent phosphorylation of Ser499 of Rap1GAP negatively regulates RapGAP activity. Sequence analysis of SIPA1L2 revealed a high scoring PKA phosphorylation motif (S990-RxxpS motif/0.757; Supplementary Fig. 1d) that is located close to the region that corresponds to the regulatory PKA sites (pSer 499) of Rap1GAP and that is well preserved between SIPA1L2 family members (Supplementary Fig. 1c). When we tested the hypothesis that PKA in analogy to Rap1GAP might negatively regulate RapGAP activity of SIPA1L2, we found that phosphomimetic SIPA1L2-470-1025-S990D indeed hydrolyzed very little recombinant Rap1b-GTP (Fig. 6g±i), indicating a negative regulation of RapGAP activity by PKA. Collectively these data suggest that following induction of presynaptic plasticity, PKA phosphorylation of Snapin induces the dissociation of the amphisome from Dynemin and enhances its residing time at presynaptic boutons. Concomitant phosphorylation of SIPA1L2 diminishes its RapGAP activity and thereby potentially facilitates ERK signaling. 

Amphisomes activate ERK at boutons and enhance release. Amphisomes are believed to be transient intermediate organelles that in nonneuronal cells rapidly enter a degradative lysosomal
pathway. However, lysosomes are not abundant if at all present in distant axons. In primary cultures, STED imaging revealed that the lysosomal marker LAMP1 was indeed mostly absent from presynaptic boutons in line with the high association of LC3, SIPA1L2 and TrkB observed before (Fig. 7b, c). This is expected to be higher when assessing colocalization in only those boutons in which SIPA1L2 is present. Accordingly, intensities of pTrkBY515 are higher in SIPA1L2-positive boutons (Fig. 7d, f). This tight association of SIPA1L2 with TrkB and LC3 (Supplementary Fig. 6a) is expected to be higher when assessing colocalization in only those boutons in which SIPA1L2 is present. Accordingly, intensities of pTrkBY515 are higher in SIPA1L2-positive boutons (Fig. 7d, f). This tight association of SIPA1L2 with TrkB and LC3 (Supplementary Fig. 6a) is expected to be higher when assessing colocalization in only those boutons in which SIPA1L2 is present. Accordingly, intensities of pTrkBY515 are higher in SIPA1L2-positive boutons (Fig. 7d, f). This tight association of SIPA1L2 with TrkB and LC3 (Supplementary Fig. 6a) is expected to be higher when assessing colocalization in only those boutons in which SIPA1L2 is present. Accordingly, intensities of pTrkBY515 are higher in SIPA1L2-positive boutons (Fig. 7d, f). This tight association of SIPA1L2 with TrkB and LC3 (Supplementary Fig. 6a). A recent study showed that TrkB is transported on autophagosomes to the soma where it regulates gene expression. This transport requires association of the Clathrin adaptor AP2 with LC3 and DIC. Therefore, we speculated that amphisomes could constitute more persistent entities in axons where they could serve signaling functions at presynaptic boutons.
Fig. 6 cLTP prolongs dwelling time of SIPA1L2-amphisomes at presynaptic boutons. a Heterologous coimmunoprecipitation experiments using SIPA1L2-470-1025-tRFP (RapGAP-PDZ) and a phosho-deficient (GFP-Snapin-S550A) or a phosphomimetic Snapin mutant (GFP-Snapin-S550D). Both forms of Snapin communoprecipitates with SIPA1L2, but only the phosho-deficient form of Snapin communoprecipitates with DIC. b, c Snapshots in b obtained from time-lapse imaging in rat hippocampal cells overexpressing GFP-SIPA1L2 and tRFP-LC3b and labeled in vivo with Stgm-1Oyster650 after control or chemical long-term potentiation (cLTP) induction. Imaging was performed in nonconditioned, extracellular imaging buffer. Below, merge images represent traces of cotrajectories aligned with positions of presynaptic boutons (shaded blue lines). Dwelling time of the SIPA1L2-LC3b-amphisomes at boutons is represented in e and corresponding cumulative distribution diagram in f. Data represented as mean ± SEM. N numbers in e, f correspond to vessels analyzed from 11 axons (control), 14 axons (cLTP), 8 axons (H89) and 6 axons (H89 + cLTP) (one-way ANOVA on ranks with Dunn’s multiple comparison test for vesicles). g Time course of Rap1b GTP hydrolysis in the presence of 50 μM of the GTPase inhibitor GDP. h Recombinant intein-tagged SIPA1L2-470-1025 as well as SIPA1L2-470-1025-S990D (phosphomimetic SIPA1L2 mutant in a potential PKA phosphosite) were used to hydrolyze recombinant Rap1b loaded with GTP in the presence of LC3b. Quantification of four independents experiments in i (Mann-Whitney U test). Bar graphs depict data as mean ± SEM. *** indicates P ≤ 0.001; **** indicates P ≤ 0.0001.

Fig. 6d) at presynapses indicate that SIPA1L2 might be part of a signaling TrkB amphisome. We also found an extensive colocalization with AP2 in axons and synaptic boutons (Supplementary Fig. 6e–g), suggesting that SIPA1L2-TrkB amphisomes are long-range organelles that will traffic retrogradely to reach the soma. Moreover, TrkB and SIPA1L2 associate with an autophagosome enriched fraction from rat brain lysates that is also positive for LC3, Rab7 and Snapin (Fig. 7i, j). Of note, SIPA1L2 was not found in the lysosome fraction (Fig. 7i, j).

What could be the consequence of amphisome stopover at axon terminals? Local ERK activation has been linked to enhanced neurotransmitter release and presynaptic plasticity. We took advantage of a FRET-based ERK sensor that we targeted to the presynaptic compartment following fusion to Synaptophysin-1 (Fig. 8a–e; Supplementary Fig. 6h). We then imaged changes in the GFP fluorescence lifetime, the donor of the FRET pair, coinciding with the stopover of amphisomes, which we identified using either tagged versions of SIPA1L2 or LC3b. Synaptic visits were determined by kymograph analysis (Fig. 8a).

Quantification of ΔLifetimeGFP upon the arrival of the amphisome at the bouton (time 0) indeed correlated with a significant decrease in the GFP lifetime (Fig. 8b–e), indicating a higher FRET efficiency due to ERK activation and subsequent phosphorylation of the sensor. Importantly, this decrease was not observed when
the amphisome passed by a bouton without stopping (nonvisited terminals) or in axons where no amphisomal trafficking was observed during the recording time (Fig. 8c–e).

ERK activation in presynaptic plasticity results in phosphorylation of Synapsin I (Syn-I), a protein known to play a role in BDNF-mediated increase of neurotransmitter release. Ultrastructural analysis revealed no major alterations in presynaptic bouton organization and vesicle content in sia1l2−/− as compared to wt mice (Supplementary Fig. 6i–j). Quantification of ERK-dependent phosphorylation of Syn-I in hippocampal primary neurons from wt mice revealed a significant enhancement of the pSyn/Syn ratio at boutons following BDNF application (Fig. 8f, g) without changes in their number (Supplementary Fig. 6k). The BDNF-induced enhancement of the pSyn/Syn ratio was absent in sia1l2−/− neurons and could be rescued by re-expression of β-SIPA1L2 in ko neurons. However, no rescue was observed when we re-expressed the SIA1L2Δ14 mutant lacking the TrkB binding region (Fig. 8f, g).

To directly assess the effects of the activation of these molecular pathways in transmitter release, we combined unloading of FM 4-64 dyes to monitor synaptic vesicle fusion with live imaging of LC3b/TrkB trafficking and compared unloading rates at visited and nonvisited boutons (Fig. 8h–j). Analysis of the unloading rates before and after a stopover revealed a significant enhancement in the majority of the analyzed boutons visited by both TrkB-GFP (Fig. 8k) and GFP-LC3b (Fig. 8l).

Importantly, this increase was not observed in nonvisited neighboring boutons (Fig. 8m).
Discussion

Autophagosomes in neurons are formed continuously in distal axon terminals from where the autophagic flux of cargoes derived from synapses is directed towards the soma in a dynein-dependent manner. Classical stimuli like starvation have relatively little effect on neuronal autophagic flux. However, it was shown previously that synaptic activity transiently upregulates autophagy at presynaptic terminals and autophagosome formation might be therefore regulated locally in individual boutons. Although there is growing appreciation that neuronal autophagy might serve synaptic function, it has not been shown yet that it might be a mechanism used to mediate activity-dependent cargo transport.
inhibits the amphisome at axon terminals and retrograde transport, and (v) amphisome stopover occurred predominantly at synaptic boutons. Right panels show the frequency distribution of unloading rates (paired Student’s t test in k, l and Wilcoxon rank test in m). Shaded lines in kymographs (a, j) represent visited (red) and nonvisited (green) boutons. Bar graphs show mean ± SEM. n.s. stands for not significant. ***, **** indicate P ≤ 0.05, P≤0.01, P ≤ 0.001, respectively.

Fig. 9 SIPA1L2 controls trafficking and signaling of TrkB-amphisomes at boutons. Signaling amphisomes result from the fusion of active TrkB containing late endosomes with autophagosomes (1). Retrograde trafficking and signaling of these amphisomes in axons are tightly regulated by SIPA1L2, which directly binds to TrkB, Snapin, and LC3b. While the binding to Snapin serves as a linker to a dynein motor, binding of LC3b enhances SIPA1L2 RapGAP activity which negatively interferes with TrkB/Rap1-signaling and also slows down the velocity of retrograde transport. (2) The amphisome halts at single endosomes with autophagosomes (1). Retrograde transport is inhibited and lysosomal markers accumulate, levels of cathepsins, the major lysosomal proteases, remain very low compared to those in the soma. If one takes into account the extreme distances in axonal transport, amphisomes will constitute stable entities long before acquiring a lysosomal identity near the neuronal cell body. Since autophagosomes fuse with late endosomes to undergo robust retrograde transport, it is probably scarce. We found that the resulting organelle has indeed features of an amphisome with signaling properties whose trafficking and signaling capabilities are tightly controlled by SIPA1L2 and its association with TrkB, Snapin, and LC3. Intriguingly, binding of LC3 enhances RapGAP activity and thereby negatively interferes with TrkB-induced Rap signaling, which is upstream of ERK activation (Fig. 9). PKA phosphorylation of Snapin and SIPA1L2 at presynaptic sites immobilizes the amphisome at axon terminals, terminates RapGAP activity and thereby allows TrkB/Rap1 signaling that will facilitate transmitter release (Fig. 9). It was proposed previously that autophagosomes contain active TrkB complexes. In this study, we provide evidence that TrkB signaling endosomes are in fact amphisomes whose formation is likely linked to the regulation of neuronal autophagy. We propose that neuronal amphisomes are stable prelysosomal hybrid organelles with signaling capabilities based on the findings that (i) they were immunopositive for the late endosome marker Rab7 but not the lysosomal marker LAMPI, (ii) TrkB in presynaptic boutons is invariably found in close proximity to SIPA1L2 and phosphorylated at Y515, which is a crucial phosphorylation site for ERK signaling, (iii) amphisome stopover occurred predominantly at active release sites, (iv) amphisome trafficking to boutons correlated with enhanced levels of phosphorylated Syn-I and ERK activation (v) finally, enhanced transmitter release.

![Diagram of Axon and Synaptic Bouton](Image)

**Fig. 8** SIPA1L2-amphisomes activate ERK at boutons and potentiate presynaptic function. a Kymographs represent the visit of SIPA1L2-amphisomes labeled by SNAP-SIPA1L2 (**SiR647**) to a bouton identified by Sy-EKAR. b Representative heat maps depicting GFP lifetime (n.s.) over time. Lifetime% represents the frame when the amphisome reaches the bouton. Scale bar is 5 μm. c, d Quantification of GFP-ΔLifetime over time in visited and nonvisited boutons. Anamorphosis is considered as time 0. Control in d represents data from axons where no trajectories were found. N numbers represent analyzed boutons from n > 3 independent experiments. e Averaged GFP-ΔLifetime from c, d. One-way ANOVA with Bonferroni’s posthoc test; n = number of boutons. f Representative confocal images from wt and sipa1l2−/− primary neurons overexpressing Fl-SIPA1L2-mCherry, SIPA1L2-A14-mCherry or mCherry, treated with TrkB-Fc or BDNF and immunostained for Synapsin1,2 (Syn) and phospho-SynS62. White arrows show nontransfected boutons. Watts depict boutons labeled with the asterisk. Scale bar is 5 μm. g Percentage of enhancement of the pSyn/Syn ratio in BDNF-treated as compared to those in the soma. If one takes into account the extreme distances in axonal transport, amphisomes will constitute stable entities long before acquiring a lysosomal identity near the neuronal cell body. Since autophagosomes fuse with late endosomes to undergo robust retrograde transport, it is probably scarce.

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inevitably that, in the absence of autolysosome formation, amphisomes serve as signaling and sorting platforms while trafficking in a retrograde direction. This makes endosomal sorting processes at axon terminals dispensable and would give an answer to the long-standing question of why neurons transport autophagic and endocytic cargos back to the cell body for degradation instead of disposing of them locally.

It is likely that amphisomes collect additional cargo during visits at synapses that may differently determine their properties and vesicular fate while propagating retrogradely to the soma. Sustained signaling capacity and diversity will promote and integrate local synaptic function with long-range signaling while evading lysosomal degradation. Specificity for certain synaptic connections is conceivable and in the case of SIPA1L2 might come from docking to presynaptic sites via for instance PDZ domain-dependent interactions. The idea of selective amphisome signaling at subpopulations of boutons is appealing since TrkB/BDNF facilitation of presynaptic transmitter release possibly requires more sophisticated signaling than the one provided by the classical TrkB signaling endosomes that lack local signaling function and select sorting substrates to induce clustered presynaptic plasticity like it was proposed by Staras et al.52. This study demonstrated the existence of a vesicle pool that is not confined to a synapse but spans multiple terminals. Vesicles within this superpool are highly mobile and are rapidly exchanged between terminals. Focal BDNF application suggests the involvement of a local TrKB-receptor-dependent mechanism for synapse-specific regulation of presynaptic vesicle pools through control of vesicle release and capture to or from the extrasynaptic pool52. Thus, clustered presynaptic plasticity is another conceivable function of amphisome signaling. This is also interesting in light of several studies that have shown that MF LTP is expressed presynaptically and requires cAMP–PKA signaling53. Our results suggest that the SIPA1L2 amphisome is a likely substrate for PKA-dependent LTP. Along these lines, we speculate that at MF synapses SIPA1L2-related amphisome signaling is important for the maintenance of LTP, which in turn requires BDNF/TrkB signaling and is important for pattern separation.

Several adaptors have been described for antero- and retrograde transport of TrkB18,34,54–57. Many of these adaptors show also an association with autophagosomes58–60 and synergies likely exist between adaptors. Interestingly, Snapin is reportedly also an adaptor for axonal lysosomes39, which further supports the idea of a single transport complex slowly acquiring a lysosomal identity during retrograde movement. We could show that SIPA1L2-mediated ride-on service for TrkB-containing amphisomes enables local and long-distance signaling (Fig. 9). In this context, it should be mentioned that other SIPA1 family members exhibit a similar domain organization with a high degree of similarity in the RapGAP, PDZ and CC-leucine zipper domain (Supplementary Fig. 1). It is thus very likely that all of them will bind Snapin and LC3 while they might differ in cargo selection for retrograde transport of amphisomes but also additional functions of the SIPA1L family in autophagy are conceivable.

Methods

Animals. Animals were maintained in the animal facility of the Leibniz Institute for Neurobiology, Magdeburg (Germany) or ZMNH, Hamburg (Germany) under controlled environmental conditions.

All animal experiments have complied with all ethical regulations for animal testing and research in accordance with the European Communities Council Directive (2010/63/EU) and were approved by the ethics committees of Sachsen-Anhalt/Germany (reference number 42502-1-1264 LIN and 42502-1-1284 UNIMID) or of the city-state of Hamburg (Behörde für Gesundheit und Verbraucherschutz, Fachbereich Veterinärwesen) and the animal care committee of the University Medical Center Hamburg-Eppendorf.

Generation of sipa1l2−/− mice. For a constitutive knockout generation, exon 2 of the Sipa2/sipa1l2 locus (Gene ID: 244666; RefSeq NM_001081337.1 (mRNA), NP_001074806.1 (protein)) was replaced by a CreRelsLacZ reporter gene and a neomycin phosphoribosyltransferase selection cassette by homologous recombination. The targeting vector was constructed by amplifying homologous regions from 129S6/Sv/Ev mouse genomic DNA using primers described in Supplementary Table 1.

Gene targeting was performed using ES cells (CCBR: 129S6/Sv/Ev strain) and chimeras were generated by injection into C57Bl/6J blastocysts. All experiments described here were performed with mice backcrossed for at least ten generations to C57BL/6J background. Genotyping was performed with primers described in Supplementary Table 1 resulting in bands of 200 bp for wt and 400 bp for knockout.

Antibodies. A list of antibodies used in this study and corresponding dilutions is available in Supplementary Table 1.

Cloning. Sources of the main constructs and primers used in this study are available in Supplementary Tables 1 and 2. For Snapin knockdown experiments, a published Snapin KD sequence58 was cloned into the pSIH-H1 shRNA vector (System Biosciences). Inserted sequences are described in Supplementary Table 1.

The Sy-EKAR vector was created by PCR amplification of EKAR-GFP:RFP (ref. 37; Addgene #18680) to which a 4Gly linker domain (GGTGGCGGTGGA) was incorporated in the N-terminus (see Supplementary Table 1). This was subcloned into the CMV: ratSyGCaMP2 (Addgene #26124), from where GCaMP2 was removed. SIPA1L2 mutants were generated using the Q5 site-directed mutagenesis kit (NEB).

Yeast Two-Hybrid. The Yeast-Two-Hybrid assay was performed as described in ref. 62.

Electrophysiology in acute hippocampal slices. MF-LTP induction was performed as described in ref. 11. Hippocampal slices from 11–16-week-old C57Bl/6J or sipa1l2−/− mice were cut and preincubated for 3 h prior recordings. When required, slices were preincubated for 3 h before recording in carbogenated ACSF, either with 1 µM of TAT-SIPA1L2 peptide or with 1 µM of TAT-scrambled peptide as a control, as well as with or without 5 µg/ml TrkB-Fc.

For assessment of postsynaptic DG LTP, field-EPSPs were measured with an electrode positioned at the middle of the molecular layer of the DG. The medial perforant path was stimulated with biphasic constant current pulses (0.1 ms per half-wave duration). Different theta-burst stimulation protocols (TBS) or a high-frequency stimulation (HFS) protocol were used for induction of synaptic plasticity. The TBS protocols consisted of either one episode of brief presynaptic bursts (ten pulses of 0.2 ms per half-wave duration delivered @10 Hz) repeated ten times @5 Hz, two of these episodes (inter-episode interval 10 s) or four episodes (inter-episode interval 10 s) repeated four times every 5 min. The HFS protocol consisted of four trains of 100 pulses (0.2 ms per half-wave duration) @100 Hz, inter-train interval 5 min.

Behavioral tests. The behavioral spatial pattern separation task was performed as described11 with minor modifications. For object location and novel object recognition task, two equal objects were placed at the equidistant position from the walls of the arena, and the animals were free to explore them. Twenty-four hours later, novel location recognition was assessed as one of the familiarized objects was displaced to a new location and mice were again left free to explore both objects over 20 min. On the third day, a novel object recognition test took place as one of the familiarized objects was now exchanged for a novel object. Animals were again left for 20 min to explore the objects before being returned to the home-cage. In experiments involving rotarod, animals were tested on an accelerating rotarod apparatus two trials per day, for 5 consecutive days, in which the speed of the rod was gradually increased each day (from 15 to 36 rpm). On day 5, another trial was submitted. Four trials lasting 300 s each, during which the rotation speed gradually increased from 4 to 40 rpm. The latency to fall was recorded.

In experiments requiring infusion of TAT-peptides in DG, animals (wt) underwent sample phase training of the spatial pattern separation task, and 5 min after the session received an intra-DG infusion of either TAT-SIPA1L2 peptide or TAT-scrambled conjugated with fluorescein. Anesthesia was induced at 5% isoflurane in O2:N2O mixture (Rothacher Medical GmbH, Switzerland) and mice were placed in the stereotaxic frame (World Precision Instruments). During surgery, anesthesia was maintained at 1.5–2.0%. Craniootomy was done targeting the dorsal dentate gyrus with stereotaxic coordinates, anteroposterior (AP) −2.0 mm, mediolateral (ML) ±1.4 mm from Bregma and dorsoventral (DV) −1.6 mm (from brain surface). Each mouse received 1.5 µl bilateral infusion of a respective peptide at a flow rate of 0.5 µl/min. The effect of TAT-peptide infusion was assessed 24 h later in the choice phase of pattern separation task. After the completion of the task, mice were deeply anesthetized and perfused transcardially with PBS and 4% paraformaldehyde (PFA) in PBS solution to verify DG localization of injections.
Cell culture, transfections and immunostaining. Maintenance and transfection of MRC5, HEK293T and COS-7 cells were performed as described in ref. 63. MRC5 cells were obtained from Leibniz Institute DSMZ (Cat#ACC36; RRID: CVCL_0063); COS-7 cells were obtained from KCLB (Cat#10171; RRID: CVCL_0224) and MRC-5 cells were obtained from KCLB (Cat#10171; RRID: CVCL_0063). Costainning with the secondary fluorochrome 123 was gained by 900 pulses @10 Hz in the presence of AP-5 (50 µM) and CNQX (10 µM) and dye unloading was imaged at a frequency of 1 Hz for 110 min. Ten frames were acquired as a baseline before stimulation. Stimulation of either TrkB-GFP or GFP-LC3b was recorded immediately after the stimulation for an additional 10 min @1 Hz. After that, a second dye unloading protocol was delivered and imaged as described.

Imaging was performed at 37 °C 5%CO2 in the VisiScope TIRF/FRAP imaging system from Visitron Systems based on Nikon Ti-E as described above. Laser lines of 488 nm were used to activate the respective fluorochrome. Time-lapse imaging of neuronal axons was acquired in a Leica TCS SP5 system controlled by Leica LAS AF software using HCX PL APO 63×1.40. Areas of ~60 × 15 µM (512 × 128 pixels) were scanned with 488, 700 and 633 nm laser lines. Fluorescence was collected using three Hyd detectors.

For experiments cLTP induction was required, neurons in growing media were treated with Rolipram (0.1 µM) and Forskolin (50 µM) for 10 min and cells were imaged after media replacement with EIB. When needed, H89 (10 µM) was added for 1 h before proceeding with the cLTP induction.

Dwelling time of the amiphost in boutons was collected from kymograms by drawing a vertical line using Fiji in trajectories that overlapped with the synaptic marker and the number of pixels was quantified. Only those stopovers where the overlap occurs for >3 vertical pixels were considered. Instant velocity was calculated from the angle of change in connectivity obtained by marking the cell soma or axonal tip during imaging.

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Imaging and quantification of pSyN/Syn ratio. Hippocampal primary cultures from wt and sipai12/-/- mice were transfected at 10 DIV with either Fl-sIPAI12-GFP or Sipa1L2-N705A-GFP were incubated in growing media in the presence of an antibody anti-Synaptotagmin1 coupled to Oyster 660 (1:250) for 1-2 h at 37 °C. Imaging was performed for 5 min at 1 Hz at 37 °C 5% CO2 on a Leica TCS SP5 system controlled by Leica LAS AF software using HCX PL APO 63×1.40. Areas of ~60 × 15 µM (512 × 128 pixels) were scanned with 488, 700 and 633 nm laser lines. Fluorescence was collected using three Hyd detectors.

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where $V$ is the concentration of buffer in millimolar (mM), $N_{\text{Prev}}$ is the total number of particles, and $N_{\text{Fh}}$ is the number of free particles. The clearances were calculated at 18°C in the presence of buffer for 5 min at 105 × g.

### Lipid raft isolation

Cerebella were homogenized in lysis buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 3% Triton X-100, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)) for 1 h at 4°C. The lysate was cleared by centrifugation incubated with anti-GFP/myc-antibody-coated magnetic beads (MultiMacs GEP Isolation Kit #130-094 253 or Miltenyi Biotec GmbH, Germany). The coimmunoprecipitation was performed in the presence of Triton X-100.

### Pull-down assays

For interaction assays between Sipa1L2 and TrkB, the matrix (amyllose-MBP) was overlaid with 6 ml ice-cold 30% sucrose and then again overlaid with 3 ml ice-cold 2× SDS sample buffer. Samples were washed with TBS-T buffer (TBS, pH 7.4, 5 mM MgCl₂, EDTA-free protease inhibitors (Complete, Roche)) and incubated with purified 6×His-tag LC3b for 120 min at 4°C in TBS buffer either alone or in the presence of purified 6×His-Snapin. Samples were then washed with TBS-T buffer (TBS, protease inhibitor, 0.1% Triton X-100). All samples were eluted with 2× SDS loading buffer and an equal amount of samples were used for SDS-PAGE.

### Rap1-GAP activity assay

Rap1-GAP activity was assayed using Active Rap1 detection kit (Cell Signaling) following the manufacturer’s instructions. Briefly, the assay employs Rap1-binding domain (RBD) of RaLgDS as an activation-specific probe for Rap1. Fused to GST-RaLgDS-RBD allows to pull down the amount of nonhydrolyzed Rap1 in the presence of RapGAP. For experiments involving overexpression of Sipa1L2, RapGAP and LIR-Sipa1L2 mutants of RaLgDS pull down of GTP-bound Rap1 was performed from HEK293T cell extracts. For experiments involving LCK and PKA phosphorylation of Sipa1L2-S990D, Intein-Sipa1L2-470-838, Sipa1L2-470-1025, Sipa1L2-470-1025-S990D, 6×His-tagged Rap1 was incubated for 120 min at 4°C in TBS buffer either alone or in the presence of purified 6×His-Snapin. Samples were then washed with TBS-T buffer (TBS, protease inhibitor, 0.1% Triton X-100). All samples were eluted with 2× SDS sample buffer, subjected to SDS-PAGE and immunoblotting. For pull-down assay of Sipa1L2 with endogenous LC3, whole brain extract was incubated with GST-Rap1-GAP, GST-Sipa1L2-RapGAP, GST-Sipa1L2-RapGAP, GST-Sipa1L2-RapGAP, as well as with GST alone. Immunodetection was done with anti-LC3b antibodies. Representative blots are shown uncropped and unprocessed in the Source Data file.

### Preparation of autophagosomal-enriched fraction

A whole brain homogenate from two rats was subjected to density gradient separation and purification as already described with minor modifications. Briefly, rat brains were homogenized and subjected to differential centrifugation through Nycodenz gradient (15, 20, 24, 26, 85%) for 3 hr at 105,000 × g. Each fraction (A1 – 15%, A2 – 20–24% and L – 24–26% interfaces) was collected, diluted with Percoll and centrifuged at 4°C for 3 hr at 105,000 × g to obtain a virtually pure pellet and analyzed using antibodies against Sipa1L2, TrkB, Snapin, LC3b, and Rab7.
Statistical analysis. In the manuscript, the data are shown as mean ± SEM and n numbers used for statistics are depicted in each panel or figure legends from at least three independent experiments. Graphs and statistical analysis was made with GraphPad Prism (GraphPad Software). Statistical tests used are given in the figure legend of the corresponding experiment. Briefly, D’Agostino–Pearson omnibus normality tests were performed to assess the normality of samples. Subsequently, parametric and nonparametric tests were chosen accordingly. For comparison between normally distributed samples, two-tailed unpaired Student’s t test was used for comparing two groups and one-way ANOVA with Bonferroni’s posthoc test was used for comparison between more than two groups. For not normally distributed samples, Mann–Whitney U test or Kolmogorov–Smirnov test were used to compare two groups and Kruskal–Wallis with Dunn’s correction was used for comparing not normally distributed samples. P values <0.05 were considered significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data supporting the findings described in this study are available from the corresponding author upon reasonable request. Source data from all representative blots shown in this study as well as data underlying all quantitative analysis performed in this study are provided with the manuscript as a Source Data file.

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M.A.-A., C.S., A.K., M.R.K. designed the study. M.A.-A., A.K., M.R.A., I.B., G.A.S., G.M.G., R.R., P.T., M.B., J.L.-R., T.J.H., T.M., C.S. conducted the experiments. M.A.-A., A.K., M.R.A., I.B., G.A.S., G.M.G., P.Y., M.B., J.L.-R., S.D.-G., M.S., T.M., C.S., M.R.K. analysed the data. O.S., N.B., M.C., A.V.F., S.A.R., M.S., M.K., E.D.G., M.R.K., provided tools and reagents. M.A.-A., A.K. and M.R.K. wrote the paper and all authors commented and revised the manuscript.

Competing interests
The authors declare no competing interests.

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