Retrograde transport of TrkB-containing autophagosomes via the adaptor AP-2 mediates neuronal complexity and prevents neurodegeneration

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Autophagosomes primarily mediate turnover of cytoplasmic proteins or organelles to provide nutrients and eliminate damaged proteins. In neurons, autophagosomes form in distal axons and are trafficked retrogradely to fuse with lysosomes in the soma. Although defective neuronal autophagy is associated with neurodegeneration, the function of neuronal autophagosomes remains incompletely understood. We show that in neurons, autophagosomes promote neuronal complexity and prevent neurodegeneration in vivo via retrograde transport of brain-derived neurotrophic factor (BDNF)-activated TrkB receptors. p150Glued/dynactin-dependent transport of TrkB-containing autophagosomes requires their association with the endocytic adaptor AP-2, an essential protein complex previously thought to function exclusively in clathrin-mediated endocytosis. These data highlight a novel non-canonical function of AP-2 in retrograde transport of BDNF/TrkB-containing autophagosomes in neurons and reveal a causative link between autophagy and BDNF/TrkB signalling.
Autophagy is an evolutionary conserved process that serves to provide nutrients during starvation and to eliminate defective proteins and organelles, such as mitochondria and the endoplasmic reticulum via lysosomal degradation. During autophagy portions of the cytoplasm are sequestered within double- or multilaminar vesicles termed autophagosomes. These undergo subsequent maturation steps, in particular fusion with late endosomes, to become late-stage autophagosomes also called amphisomes before being delivered to lysosomes by dynein-mediated retrograde transport. Autophagosome formation requires an E3-like complex comprising ATG5 that catalyses lipid conjugation of microtubule-associated protein 1 light chain 3 (LC3) (ref. 2).

In addition to the cytoprotective function of autophagy under conditions of starvation, recent data support additional roles of autophagy, for example, in maintenance of stemness or FGF signalling to mediate bone growth during development. In the brain, autophagosomes form locally in distal axons and are trafficked retrogradely to eventually fuse with lysosomes enriched in the neuronal soma. Accumulation of autophagosomes is a hallmark of neurodegenerative disorders including Alzheimer’s and Huntington’s disease, or amyotrophic lateral sclerosis, while knockout (KO) of key autophagy proteins in mice causes neurodegeneration. In spite of these findings the physiological function of neuronal autophagosomes and their role in promoting neuronal survival and counteracting neurodegeneration remains incompletely understood.

A crucial pathway that promotes neuronal survival, protects from neurodegeneration and promotes neuronal complexity is the brain-derived neurotrophic factor (BDNF) signalling pathway. In cortical and hippocampal neurons, BDNF initiates signalling by binding to its receptor TrkB in distal neurites. Activated BDNF/TrkB complexes are internalized predominantly via macropinocytosis mediated by EHD4/pincher into so-called ‘signalling endosomes’ that are refractory to lysosomal degradation to ensure persistent signalling. Consistent with this model, BDNF/TrkB have been shown to require retrograde axonal transport to promote neuronal branching and survival and to counteract neurodegeneration. Recent data suggest that TrkB-signalling endosomes may contain late endosomal markers such as Rab7 and are trafficked in part by Snapin, a subunit of the BLOC-1 complex. KO mice lacking Snapin suffer from impaired neurosecretion, but do not show major defects in brain architecture or neuronal complexity associated with defective BDNF/TrkB signalling, suggesting that other factors must exist that promote retrograde traffic of TrkB-signalling endosomes. However, neither the identity of these factors nor the cell biological nature of TrkB-signalling endosomes is known.

Here we demonstrate that TrkB-signalling endosomes are late-stage autophagosomes that undergo retrograde transport to the neuronal soma via their association with the adaptor AP-2, an essential protein complex hitherto thought to function exclusively in clathrin-mediated endocytosis and in the reformation of synaptic vesicles in the brain. AP-2 is a heterotetramer comprised of α, β, μ, and σ subunits. We show that neuronal AP-2 mediates retrograde transport of TrkB-containing autophagosomes via association of AP-2α with LC3 and of AP-2β with the p150Glued subunit of the dynnein cofactor dynactin to promote neuronal complexity and counteract neurodegeneration in vivo. Our data, thus, identify a novel function of autophagy in BDNF/TrkB signalling during brain development mediated by a non-canonical role of the endocytic adaptor AP-2.

Results

Retrograde co-trafficking of AP-2 and LC3 in neurons. The localization and function of neuronal AP-2 so far has been investigated largely by analysis of its steady-state distribution in fixed neurons and brain tissue or by genetic and biochemical experiments. Recent data support the dynamics of fluorescent protein-tagged AP-2α (tagged at an internal site that retains full functionality) by live imaging of primary cortico-hippocampal neurons in culture. In addition to the plasma membrane, AP-2α-mRFP localized to puncta in axons and dendrites (Fig. 1a,b). In axons, AP-2α-mRFP-positive puncta underwent rapid bidirectional movement (at 0.4–0.5 μm s⁻¹) (Fig. 1c, Supplementary Movie 1) suggestive of microtubule-based transport with a preference for retrograde transport to the neuronal soma (Fig. 1d). In contrast, no retrograde transport of AP-2α-mRFP was seen in dendrites (Supplementary Fig. 1a,b, Supplementary Movie 2). Given the reported association of AP-2 with autophagic proteins and the prominent microtubule-based movement of autophagosomes in neurons, we hypothesized that mobile AP-2-containing structures may correspond to LC3-positive autophagosomes. Live cell image analysis revealed a close colocalization (Supplementary Fig. 1c,d) and co-trafficking (Fig. 1e,f, Supplementary Movie 3) of eGFP-LC3b with AP-2α-mRFP in primary neurons. Pearson’s correlation analysis showed that the degree of colocalization between AP-2α-mRFP and eGFP-LC3 (R₂ = 0.65 ± 0.05) (Fig. 1g) was comparable to that of eGFP-LC3 with mCherry-ATG12 (R₂ = 0.67 ± 0.02), a bona fide component of the machinery for autophagosome formation (Supplementary Fig. 1e,f). In agreement with previous reports, we found that >80% of stationary AP-2 puncta in the axons were confined to synapses (Supplementary Fig. 1g–i). Partial co-localization of endogenous AP-2 with LC3 in neuronal processes was further confirmed by dual-colour time-gated stimulated emission depletion microscopy (STED) analysis of neurons treated with the vATPase inhibitor folimycin, a lysosomotropic agent, which prevents autophagosome degradation and, thus, LC3b degradation via lysosomal proteolysis, and immunostained for AP-2α and LC3b (Fig. 1h,i, Supplementary Fig. 1j,k). In clathrin-mediated endocytosis, AP-2 functions to recruit clathrin and cargo proteins to endocytic sites at the plasma membrane. Surprisingly, we failed to detect a specific enrichment of clathrin on AP-2-positive autophagosomes (Supplementary Fig. 1l,m). These data suggest that retrograde co-trafficking of AP-2 on autophagosomes may reflect a novel non-canonical function of AP-2 in autophagosome transport in primary neurons that is likely independent of its established role in clathrin-mediated endocytosis as further discussed below.

AP-2 forms a complex with LC3 and dynactin/p150Glued. The co-trafficking of AP-2 with LC3 on autophagosomes raises the question how neuronal AP-2 is recruited to these carriers. While the β, μ and σ subunits of AP-2 complex are made from a single gene in mammals, the α subunit is encoded by two isogenes termed αA and αC that undergo alternative splicing in the brain. Recent data suggest that AP-2 can associate with LC3 via a putative LIR motif within the appendage domain of the AP-2α subunit. To probe whether AP-2 via its α appendage domain directly binds to LC3 we carried out binding assays using purified proteins (Fig. 2a, Supplementary Fig. 2a,c). Purified recombinant LC3 was found to bind to the GST-tagged appendage domains of both AP-2α and AP-2αC with a preference for AP-2αA over AP-2αC (Fig. 2a). In line with this, endogenous AP-2αA/C co-purified with GST-LC3b (Supplementary Fig. 2c) in affinity
chromatography experiments from brain lysates (Fig. 2b), while, conversely, GST-AP-2α and, less well, GST-AP-2β associated with native LC3b (Supplementary Fig. 2b). Consistent with the preferential retrograde transport of AP-2-containing LC3-positive autophagosomes, we found endogenous AP-2 to co-immunoprecipitate with the p150Glued subunit of dynactin, a cofactor for the retrograde microtubule-based motor dynein, but not with the anterograde trafficking motor Kif5A from detergent-extracted rat brain lysates (Fig. 2c). Moreover, p150Glued expressed in HEK293T cells was able to capture the purified appendage domain of AP-2β in binding assays (Fig. 2d). Finally, affinity purification of endogenous AP-2 using GST-fused Stonin 2 as a bait resulted in the co-purification of both LC3b and p150Glued (Fig. 2e, see also Supplementary Fig. 2d), suggesting that all three proteins act as part of a complex. Consistent with these biochemical data we observed the close colocalization of endogenous LC3b with p150Glued and AP-2 in neurons treated with folimycin (Supplementary Fig. 2e–h). Collectively, these findings indicate that AP-2 associates with LC3 and p150Glued in a protein complex that may mediate retrograde transport of autophagosomes.

**AP-2 regulates retrograde autophagosome transport in neurons.** To test this hypothesis we probed whether AP-2 is functionally important for retrograde autophagosome transport by conditionally ablating expression of the essential AP-2β subunit in neurons25. Tamoxifen addition to DIV0 cultured hippocampal neurons isolated from newborn mice carrying floxed alleles of AP-2β and expressing a tamoxifen-inducible Cre recombinase

![Figure 1](image1.png)
(AP-2\(\mu^{\text{lox/lox}}\cdot\text{CAG-iCre}\)) resulted in strongly reduced expression of neuronal AP-2 (monitored by its \(\alpha\) subunit) (Supplementary Fig. 3a,b). The levels of other endocytic or presynaptic proteins analysed in brain lysates from conditional neuronal-confined AP-2 KO neurons (AP-2\(\mu^{\text{lox/lox}}\cdot\text{Tub}\_1\)-Cre mice; described in more detail below) were largely unaffected except for a small, yet statistically insignificant reduction of synaptotagmin 1 (Supplementary Fig. 3c,d), an established AP-2 binding partner31. We then monitored the transport of autophagosomes in wild-type (WT) and AP-2\(\mu\) KO neurons expressing mRFP-eGFP-LC3 by live imaging. In WT neurons mRFP-labelled autophagosomes displayed bidirectional movements with an average retrograde velocity of about 0.4–0.5 \(\mu\text{m s}^{-1}\) (Fig. 3a–c), similar to the values obtained for AP-2\(\mu\)-mRFP with which it colocalizes (compare Fig. 1) and consistent with earlier data10,32. AP-2\(\mu\) deletion greatly reduced retrograde autophagosome velocity and the mobile fraction of retrograde LC3b-positive carriers (Fig. 3a–c, Supplementary Fig. 3e), while the fraction of stationary LC3b puncta was increased (Supplementary Fig. 3f). In agreement with the function of dynein motors in slow anterograde movement, lack of neuronal AP-2 caused a mild, yet statistically insignificant reduction in anterograde autophagosome transport (Supplementary Fig. 3g). Transport of mitochondria proceeded unperturbed in absence of neuronal AP-2 (Supplementary Fig. 3h–k). These data suggest that AP-2 regulates retrograde transport of autophagosomes from neurites to the cell soma, where most lysosomes are located5,10. Consistent with this hypothesis and with our live imaging results analysis of AP-2\(\mu\) KO neurons by thin-section electron microscopy revealed an accumulation of dense vesicular and concentric multilamellar organelles.
Figure 3 | AP-2 regulates autophagosome transport in neurons. (a) Time-lapse images of mRFP-LC3-positive puncta (arrows) in WT and AP-2 KO neurons. Scale bar, 5 μm. (b) Kymographs of mRFP-LC3 carriers generated from (a). (c) Average retrograde velocity of mRFP-LC3 carriers in WT and AP-2 KO neurons. Loss of AP-2 significantly decreased the LC3 velocity compared to WT controls (WT: 0.44 ± 0.07 μm s⁻¹, KO: 0.21 ± 0.03 μm s⁻¹, *P = 0.019, n = 4 independent experiments, 49–67 neurites per condition). (d-e) Electron micrographs of synapses from cultured WT and AP-2 KO neurons. AP-2 KO neurons accumulate dense vesicular bodies with the majority representing concentric multilamellar structures (black boxes in d represent magnified areas in e). Scale bars, (d) 500 nm, (e) 100 nm. Sp, spine. See also Supplementary Fig. 3l-o. (f) Percentage of WT and KO synapses containing dense vesicular bodies (WT: 6.00% ± 1.54%, AP-2 KO: 13.00% ± 2.52%, *P = 0.045, n = 4, 100 synapses per condition). (g-i) Representative confocal images of cultured WT and AP-2 KO neurons immunostained for LC3b and Rab7 (white boxes in g represent the magnified areas in h,i). Scale bars, (g) 15 μm, (h,i) 2 μm. (j,k) Accumulation of LC3b-containing structures (LC3b puncta μ⁻²) are depicted, WT: 0.009 ± 0.002, AP-2 KO: 0.040 ± 0.009, *P = 0.016, n = 4, in total 39 AP-2 KO and 33 WT neurons) (j) and Rab7-containing structures (Rab7 puncta μ⁻²) are depicted, WT: 0.004 ± 0.000, AP-2 KO: 0.007 ± 0.000, *P = 0.042, n = 3, in total 29 AP-2 KO and 23 WT neurons) (k) in AP-2 KO neurons. (l) Enhanced colocalization of LC3b with Rab7 on neuronal autophagosomes in absence of AP-2 based on Pearson’s coefficient (Rp) (WT: 0.52 ± 0.04, AP-2 KO: 0.64 ± 0.01, *P = 0.032). Rp was calculated for 64–84 regions of interest (ROI) per condition from three independent experiments (n = 3). (m,n) Bar diagrams indicating similar numbers of LC3b- (WT: 0.09 ± 0.01, AP-2 KO: 0.1 ± 0.02) (m) and Rab7-positive puncta μ⁻² (WT: 0.05 ± 0.02%, AP-2 KO: 0.04 ± 0.01) (n) in WT and AP-2 KO neurons treated with folimycin. Shown is the number of puncta per μm² (n = 3 independent experiments, 26 neurons per condition). Data in c,f–n are illustrated as box plots as described in Methods. Data reported in the text are mean ± s.e.m. NS, non-significant.
resembling late-stage autophagosomes (also termed amphisomes) post-fusion with late endosomes (Fig. 3d–f, Supplementary Fig. 3o and below). As reported earlier\(^6\), AP-2\(\mu\) KO neurons displayed a reduced number of synaptic vesicles (to about 60% of those seen in WT), in agreement with its canonical function in synaptic vesicle reformation. The 'spheroid-like' accumulation of late-stage autophagosomes in neurites and in the soma of AP-2\(\mu\) KO compared to WT control neurons was confirmed by immunostaining with antibodies against endogenous LC3b and late endosomal Rab7 (Fig. 3g–i for quantifications), indicating that the autophagosomal structures observed by light and electron microscopy have undergone fusion with late endosomes. In contrast, no significant alterations in the number or localization of early endosomes marked by Rab5 (Supplementary Fig. 4a,b) or LAMP1-positive late endosomes/lysosomes (Supplementary Fig. 3j–n, Supplementary Fig. 4c,d) were observed. To probe whether loss of AP-2 may alter LC3b synthesis and/or degradation\(^34,35\), we treated WT and KO neurons with the degradative substrate p62/SQSTM1 (Fig. 3m, Supplementary Fig. 4e). Thus, LC3b accumulation in absence of AP-2 does not appear to result from increased LC3b synthesis. A similar increase in folinycin-treated AP-2 KO neurons was observed for Rab7-positive puncta (Fig. 3n). These results argue that the accumulation of late-stage autophagosomes in AP-2 KO neurons is caused by decreased degradation of LC3b/Rab7-positive autophagosomes due to their defective retrograde transport.

**AP-2 regulates autophagy independent of endocytosis.** As decreased degradation of LC3b/Rab7-positive autophagosomes in the absence of AP-2\(\mu\) might result from their defective delivery to lysosomes, we next probed the turnover of autophagosomes in the absence of AP-2\(\mu\) using mRFP-eGFP-LC3 as a reporter (Fig. 4a). Serum deprivation caused an elevation of the mRFP/eGFP fluorescent ratio in WT neurons indicative of increased starvation-induced autophagic flux. In contrast, no significant changes in the mRFP/eGFP ratio were observed in AP-2\(\mu\) KO neurons (Fig. 4b, Supplementary Fig. 4f). Moreover, AP-2 loss resulted in the accumulation of the autophagic adaptor and degradative substrate p62/SQSTM1 (Fig. 4c,d) that persisted upon inhibition of protein synthesis by cycloheximide (Supplementary Fig. 4g), suggesting that it is the result of defective autophagosome turnover. Consistent with this, mTORC1 signalling measured by phospho-S6 kinase 1 and phospho-Raptor levels was not significantly changed in AP-2 KO brains, although we detected a slight increase in the total amount of S6 kinase (Supplementary Fig. 4h,i). These data suggest that autophagosome accumulation in absence of AP-2 is not a consequence of reduced mTORC1 activity, a key repressor of autophagosome formation.

Our results described thus far indicate that loss of AP-2 causes defective retrograde transport and accumulation of autophagosomes in neurons, likely via its association with p150\(^{Glu\text{d}}\) and with LC3. A prediction from this hypothesis is that AP-2 binding to LC3 is required for autophagosome transport and turnover. To test this directly we capitalized on a mutant variant of AP-2\(\alpha\) that due to mutational inactivation of its LIR motif fails to associate with LC3 (ref. 29). Overexpression of LC3 binding-deficient mutant, but not WT AP-2\(\alpha\) in control neurons phenocopied AP-2\(\mu\) loss with respect to defective mRFP-eGFP-LC3 conversion and retrograde autophagosome transport (Fig. 4e,f). By contrast, mutant AP-2\(\alpha\) was perfectly capable of restoring defective clathrin-mediated endocytosis of transferrin in HeLa cells depleted of endogenous AP-2\(\alpha\) and AP-2\(\mu\) (Fig. 4g,h). Collectively, these data reveal a novel function for AP-2 in retrograde axonal transport of LC3/Rab7-containing autophagosomes in neurons that appears to be independent of its established role in endocytosis.

**Autophagosome transport by AP-2 promotes neuronal complexity.** What is the function of LC3/Rab7-containing autophagosomes trafficked retrogradely via complex formation of AP-2 with LC3? Previous data show that Rab7-positive organelles act as retrograde shuttling devices for active neurotrophins including BDNF and its main receptor TrkB (refs 36,37) to mediate nuclear signalling\(^38\) and neuronal arborization\(^39\). To test this, we monitored the retrograde dynamics of TrkB-mRFP by live imaging. TrkB-mRFP was present on both large, as well as smaller diffraction-limited mobile puncta within the soma and in neurites of BDNF-stimulated neurons, where it colocalized with eGFP-LC3-positive autophagosomes (Fig. 5a,b; Supplementary Movie 4). In the absence of BDNF the mobility of TrkB-mRFP puncta was greatly reduced in WT (Fig. 5c,d), but not in AP-2 KO neurons (Supplementary Fig. 5a,b), suggesting that BDNF may induce the targeting of signalling-active TrkB to autophagosomes for AP-2/LC3/p150\(^{Glu\text{d}}\)-mediated transport. Consistent with this hypothesis we observed that TrkB-mRFP, as well as the active phosphorylated form of TrkB (pTrkB) and the TrkB signalling component growth factor receptor-bound protein 2 (Grb2) accumulated in LC3b-containing autophagosomes in the absence of AP-2\(\mu\) (Fig. 5e–g, Supplementary Fig. 5c–f). The levels of full-length TrkB and its truncated isoform T1 were elevated in brains of conditional AP-2\(\mu\) KO mice (Supplementary Fig. 5g,h), which persisted upon inhibition of protein synthesis by cycloheximide (Supplementary Fig. 5i,j), suggesting that it is the consequence of impaired turnover of stalled TrkB-containing autophagosomes. The levels of the p75NGF receptor, which can also be activated by BDNF, but is not coupled to the BDNF/TrkB signalling pathway were unaltered (Supplementary Fig. 5k–m). Importantly, AP-2\(\mu\) loss significantly reduced the mobility of TrkB-mRFP puncta (Fig. 5h–j), in line with their retrograde traffic via autophagosomes. Endocytosis of active TrkB proceeded in the absence of AP-2\(\mu\) (Supplementary Fig. 5n–p), consistent with TrkB being internalized predominantly via EHD4/pincher-mediated macropinocytosis\(^18\) and with an endocytosis-independent role of AP-2 in retrograde transport of TrkB-containing autophagosomes. Collectively, these data indicate that AP-2 mediates retrograde transport of LC3-positive autophagosomes containing active TrkB.

Retrograde trafficking of TrkB from axons to the neuronal soma is required for neurotrophin signalling to promote neuronal arborization and complexity\(^39\). Hence, defective retrograde transport of TrkB-containing autophagosomes in the absence of AP-2 would be expected to result in reduced neuronal complexity and impaired dendritic arborization. We directly tested this in hippocampal neurons from AP-2\(\mu\)\(^{lox/lox}\),CAG-iCre mice, in which AP-2\(\mu\) expression can be acutely abrogated by tamoxifen treatment. As predicted neuronal complexity was severely reduced in tamoxifen-treated AP-2\(\mu\) KO neurons (treated from DIV0) compared to mock-treated WT control neurons (Fig. 5k,l), a phenotype rescued by re-expression of AP-2\(\mu\) at DIV8 (Fig. 5m, Supplementary Fig. 6a–c). Moreover, expression of LC3 binding-deficient AP-2\(\alpha\) (Fig. 5n, Supplementary Fig. 6d,e) or partial depletion of the p150\(^{Glu\text{d}}\) subunit of dynactin (Supplementary Fig. 6f–i) in WT control neurons both phenocopied decreased neuronal complexity observed in neurons lacking AP-2\(\mu\), suggesting that retrograde axonal transport of TrkB-containing
Figure 4 | AP-2 regulates autophagosome turnover independent of its role in endocytosis. (a) Tandem mRFP-eGFP-tagged LC3 as a reporter of autolysosome formation. (b) Mean mRFP/eGFP intensity ratio in control or serum-deprived WT or AP-2 KO neurons (n = 6 independent experiments, with 37–54 neurons per condition). No significant difference between WT and AP-2 KO neurons was observed at steady state (P = 0.398). Serum deprivation failed to trigger the formation of autolysosomes in AP-2 KO neurons (control WT: 1.247 ± 0.092, serum-deprived WT: 2.847 ± 0.213, ***P < 0.001, control KO: 1.528 ± 0.130, serum-deprived KO: 1.774 ± 0.169, P = 0.640; serum-deprived WT versus serum-deprived KO **P = 0.002). (c) Representative confocal images of WT and AP-2 KO neurons immunostained for p62. Scale bars, 20 μm. (d) Increased number of p62-positive puncta per μm² in AP-2 KO (0.030 ± 0.003) compared to WT neurons (0.017 ± 0.003). * P = 0.046, n = 4, 33–39 neurons per condition. See also Supplementary Fig. 4g. (e) Average retrograde velocity of mRFP-LC3 carriers in control neurons expressing AP-2αWT or LC3 binding-deficient AP-2αMut and co-expressing mRFP-eGFP-LC3. Loss of LC3-AP-2α binding significantly decreased LC3 transport compared to AP-2αWT expressing controls (AP-2αWT: 0.42 ± 0.00 μm s⁻¹, AP-2αMut: 0.32 ± 0.01 μm s⁻¹, **P = 0.007, n = 3 independent experiments, ≥ 45–47 neurites per condition). (f) Mean mRFP/eGFP intensity ratio in control or serum-deprived neurons expressing AP-2αWT or AP-2αMut (n = 4 independent experiments, ≥ 25 neurons per condition). No significant difference between neurons expressing AP-2αWT or AP-2αMut was observed at steady state (P = 0.661). Serum deprivation fails to trigger autolysosome formation in neurons expressing AP-2αMut (control AP-2αMut: 1.544 ± 0.361, serum-deprived AP-2αMut: 1.775 ± 0.088, P = 0.886), but not in neurons expressing AP-2αWT (control AP-2αWT: 1.173 ± 0.091, serum-deprived AP-2αWT: 2.660 ± 0.240, **P = 0.003). (g,h) LC3-binding defective AP-2αMut restores clathrin-mediated endocytosis of transferrin in HeLa cells depleted of endogenous AP-2α (KD) (*P = 0.041, **P = 0.007, n = 4, 156, 124, 148 cells per condition, respectively). Mean grey values of transferrin uptake in KD + AP-2αWT and KD + AP-2αMut conditions were normalized to KD condition set to 100%. Scale bar, 20 μm. Tf, transferrin. Data in b,d,e,f are illustrated as box plots as described in Methods. Data in h and all data reported in the text are mean ± s.e.m. NS, non-significant.

AP-2α induced at DIV8 significantly impaired the neuronal branching complexity of mature neurons (Supplementary Fig. 6k,l). These data suggest that loss of AP-2-mediated retrograde autophagosome transport induces post-developmental neurite pruning in cultured neurons.

A prediction from these data is that defective autophagosome maturation and, thus, impaired shuttling of TrkB signals should

autophagosomes mediated by an AP-2/LC3/p150Gluad complex is required for proper neuronal arborization. To test whether the loss of neuronal branching in DIV14 neurons results from a failure in neurite development or whether it is a consequence of dendrite pruning, we induced AP-2 loss in fully developed neurons, by applying tamoxifen in cultured eGFP-expressing neurons at DIV8 (Supplementary Fig. 6i). Conditional loss of

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result in reduced neuronal complexity, akin to loss of AP-2 or p150Glued function. To test the prediction that reduced neuronal complexity is causally linked to defective TrkB-containing autophagosome transport, we conditionally deleted ATG5, an E3 ligase mediating early autophagosome expansion and maturation\(^4\), via tamoxifen-induced recombination in neurons from ATG5\(^{lox/lox}\) CAG-iCre mice (ATG5 KO). Conditional loss of ATG5 in hippocampal neurons in culture resulted in defective neuronal arborization (Fig. 5o,p, Supplementary Fig. 6m). We conclude that AP-2/LC3-mediated retrograde transport of...
TrkB-containing autophagosomes promotes neuronal complexity in vitro.

Given the prominent defects in transport of TrkB-containing autophagosomes and neuronal arborization observed in hippocampal neurons in culture following acute inactivation of AP-2β, we wanted to explore the functional importance of AP-2 for TrkB-mediated neuronal arborization in the brain in vivo. To specifically ablate AP-2β expression in CNS neurons we crossed floxed AP-2β mice with a strain expressing Cre under the neuron-specific tubulin α1 promoter beginning from embryonic day 13.5 (AP-2βlox/loxTub21-Cre mice). Conditional AP-2β KO mice were born well below Mendelian ratios (KO: 16% instead of 25% as expected, P < 0.0011, see Supplementary Fig. 7a) and lagged behind in postnatal development including cessation of weight gain at about 2 weeks and postnatal lethality between postnatal day (p) 21 and 26 (Fig. 6a,b, Supplementary Fig. 7b). To analyse the role of AP-2 in dendritic arborization in vivo we visualized the branching complexity of neurons in the cortex of p20 control and AP-2 KO mice by Golgi silver impregnation. Neuron-specific AP-2β KO mice displayed dramatic defects in dendritic architecture compared to WT littermates (Fig. 6c–f), as evident from Sholl analysis of stellate cells in cortical layers III and II, respectively (Fig. 6g,h). These data confirm our observations from cultured AP-2β KO neurons and suggest that neuronal AP-2 is required for neuronal arborization in vivo.

Neuronal AP-2 prevents neurodegeneration in vivo. Histopathological analysis of Nissl-stained brain sections from AP-2β KO mice at p20 further revealed a marked degeneration of the thalamus (Fig. 6i), including micro-vacuolations within the thalamus (Fig. 6i), including micro-vacuolations within the thalamus (Fig. 6i) and II, respectively (Fig. 6j). These data suggest that AP-2 is required to prevent neuronal loss and neurodegeneration.

A major target of TrkB signalling is BDNF, the expression of which has recently been found to be under the control of a TrkB-mediated positive feedback loop. Thus, if AP-2 loss indeed impairs TrkB signalling one would expect the expression of BDNF to be reduced in KO mice lacking neuronal AP-2. In agreement with this hypothesis, we found pro-BDNF and BDNF levels to be reduced by about 50% in AP-2 KO mice (Fig. 7b,c, Supplementary Fig. 7v,w). Given the positive feedback loop between TrkB signalling and BDNF expression, we analysed BDNF mRNA expression levels in WT and AP-2 KO neurons by qPCR (Fig. 7d). Strikingly, BDNF mRNA levels were significantly decreased in the absence of AP-2 compared to WT controls, in agreement with defective TrkB signalling due to stalled autophagosome transport in AP-2 KO neurons. We reasoned that defective arborization of AP-2β KO neurons should be rescued by boosting BDNF signalling through exogenous application of BDNF. In this setting bath application of BDNF in mass cultures of AP-2 KO neurons is expected to activate their soma-confined TrkB receptors, thereby eliminating the necessity for retrograde transport along the axon. Sustained application of BDNF indeed was sufficient to rescue defective dendritic arborization in the absence of AP-2β, while it had no effect on the number of branches in WT neurons (Fig. 7e,f).

As thalamic afferents profoundly affect postnatal development of the somatosensory cortex, we analysed the morphology of barrel compartments in WT and AP-2β-deficient mice. Barrel compartments were absent in Nissl-stained brain sections of AP-2β KO mice (Supplementary Fig. 7q), a phenotype, which was confirmed by immunostaining for potassium-chloride transporter member 2 (KCC2) (Fig. 6j). These morphological and gross anatomical alterations were accompanied by an accumulation of p62/SQSTM1 (Fig. 6k–m) and of LC3b/Rab7-containing puncta, likely corresponding to stalled late-stage autophagosomes (Supplementary Fig. 7u–w).

Impaired BDNF-TrkB signalling in AP-2-deficient neurons. The data described thus far suggest a model according to which AP-2 promotes retrograde transport of LC3/Rab7-positive autophagosomes containing active TrkB complexes to convey distal BDNF signals to the soma and thereby promote neuronal complexity (Fig. 7a). A major target of TrkB signalling is BDNF itself, the expression of which has recently been found to be under the control of a TrkB-mediated positive feedback loop. Thus, if AP-2 loss indeed impairs TrkB signalling one would expect the expression of BDNF to be reduced in KO mice lacking neuronal AP-2. In agreement with this hypothesis, we found pro-BDNF and BDNF levels to be reduced by about 50% in AP-2 KO mice (Fig. 7b,c, Supplementary Fig. 7v,w). Given the positive feedback loop between TrkB signalling and BDNF expression, we analysed BDNF mRNA expression levels in WT and AP-2 KO neurons by qPCR (Fig. 7d). Strikingly, BDNF mRNA levels were significantly decreased in the absence of AP-2 compared to WT controls, in agreement with defective TrkB signalling due to stalled autophagosome transport in AP-2 KO neurons. We reasoned that defective arborization of AP-2β KO neurons should be rescued by boosting BDNF signalling through exogenous application of BDNF. In this setting bath application of BDNF in mass cultures of AP-2 KO neurons is expected to activate their soma-confined TrkB receptors, thereby eliminating the necessity for retrograde transport along the axon. Sustained application of BDNF indeed was sufficient to rescue defective dendritic arborization in the absence of AP-2β, while it had no effect on the number of branches in WT neurons (Fig. 7e,f).
in agreement with published data\textsuperscript{50,51}. Under similar conditions nerve growth factor (NGF) application failed to rescue the reduced neuronal complexity in AP-2\textsubscript{m}-deficient neurons (Fig. 7g).

Taken together our results reveal a novel function for AP-2 in BDNF/TrkB signalling via promoting retrograde transport of TrkB-containing autophagosomes. As retrograde transport of TrkB-containing endosomes is required for neurotrophins to exert their transcriptional regulation of activity-dependent genes, most notably of BDNF (ref. 49), in the nucleus\textsuperscript{38,48}, the loss of neuronal complexity and subsequent neurodegeneration in AP-2 KO mice is likely the consequence of defective BDNF gene

\begin{figure}
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\includegraphics[width=\textwidth]{figs}
\caption{Figure depicting neuronal complexity and survival in AP-2 WT, Het, and KO mice.}
\end{figure}
transcriptional regulation due to impaired retrograde autophagosome transport (Fig. 7a).

**Discussion**

Our results reveal a novel function of autophagosomes as retrograde shuttles for TrkB-signalling complexes in neurites to promote neuronal branching and to prevent neurodegeneration. We show that TrkB-signalling endosomes, proposed to convey distal BDNF signals to the soma more than 15 years ago, may in fact correspond to late-stage LC3/Rab7-positive autophagosomes containing active TrkB complexes (Fig. 7a). In neurons, unlike non-neuronal cells, the formation of autophagosomes and their turnover by fusion with lysosomes is spatially segregated as autophagosomes form in distal axons and mature during their retrograde transport along microtubules to the cell soma, which contains the majority of lysosomes. This arrangement allows neurons to integrate TrkB/BDNF signals from their distal axons to spatiotemporally instruct neuronal branching and to allow for survival via a positive feedback loop that in turn controls BDNF expression, while active TrkB complexes may eventually undergo lysosome-mediated degradation upon their arrival in the neuronal soma. Together with the observation that distal autophagosomes have not yet been acidified and are proteolytically inactive, this predicts that premature acidification of TrkB-containing autophagosomes impairs TrkB/BDNF signalling and, thus, neuronal circuit development (consistent with ref. 18). Indeed, recent data have shown that overacidification of TrkB-containing organelles in the absence of the Christian syndrome protein NHE6, an Na+/H+ exchanger known to underlie postnatal microcephaly, intellectual disability, epilepsy and autism, results in defective neuronal arborization akin to loss of AP-2 or ATG5 reported here.

Retrograde axonal transport of TrkB-containing autophagosomes to the soma is promoted by a protein complex comprising the autophagy protein LC3, the dynein activator p150Glued and the endocytic adaptor AP-2 (Fig. 7a), a protein hitherto thought to be exclusively involved in clathrin-mediated endocytosis and reformation of synaptic vesicles. Several lines of evidence suggest that the function of AP-2 in retrograde axonal transport of TrkB-containing autophagosomes is independent of its established role in endocytosis. First, AP-2 is associated and co-trafficked with autophagosomes (Fig. 1) containing active TrkB receptors post-internalization (Fig. 5). Second, and consistent with this, we find AP-2 to be largely dispensable for BDNF-induced TrkB endocytosis in cultured neurons (see Supplementary Fig. 5n–p). Third, we show that defective retrograde transport and accumulation of autophagosomes in neurons in absence of AP-2m is phenocopied by overexpression of an LC3 binding-deficient mutant version of AP-2x that is fully functional with respect to endocytosis (Fig. 4). Finally, we demonstrate that AP-2 via distinct subunits directly associates with LC3 and with p150Glued independent of its association with endocytic proteins. The LC3-AP-2-p150Glued complex likely is of special importance in neurons, where autophagosomes are transported over large distances and with high precision and speed. A role of AP-2 in neuronal branching is supported by data from mammalian neurons and from *Drosophila* suggesting a key role for AP-2-associated kinase 1, a crucial activator of AP-2, in dendrite arborization. Interestingly, recent data have identified an endocytosis-independent role for the endocytic protein endophilin in neuronal autophagosome formation, for example, upstream of the function of AP-2 in autophagosome transport described in this study. How AP-2 switches between its functions in endocytosis and retrograde transport of TrkB-containing autophagosomes remains to be determined.

It is possible, if not likely, that additional factors besides AP-2 act as adaptors for retrograde dynen1-based motor complexes to shuttle TrkB-containing autophagosomes to the neuronal soma. These include the Snapin subunit of BLOC-1, which has been postulated to bind to dynen2 or JIP1, a multifunctional adaptor implicated in both antero- as well as retrograde movement of autophagosomes. These factors could either cooperate with AP-2, for example, to coordinate recruitment of both dynen and p150Glued/dynactin, or else distinct retrograde adaptors may operate in distal versus proximal axons. Future studies will be needed to distinguish between these possibilities. Irrespective of the precise molecular mechanisms the identification of autophagosomes as shuttling devices to convey TrkB/BDNF signals to the neuronal soma adds a new facet to the functions of autophagy. Based on our results we predict that autophagy-inducing agents such as polyamines may act as potent therapeutics for the treatment of neurodegenerative and age-associated nervous system disorders, that are often known to be associated with defective axonal transport by mediating TrkB/BDNF signalling.

**Methods**

**Animals.** Animals were housed in small groups on a 12h light/dark cycle with food and water *ad libitum*. All animal experiments were approved by the ethics committees of the LAGeo Berlin and LANUV Cologne and were conducted according to the committee’s guidelines. Wistar rats for the experiments presented in Fig. 2c and Supplementary Fig. 6i were obtained from the animal facility of the Mossakowski Medical Center of the Polish Academy of Sciences (Warsaw, Poland). Conditional AP-2 KO (AP-2lox/lox × inducible CAG-Cre (ref. 60) and AP-2lox/lox × Tubulin1 Cre) mice were described previously, ATG5lox/lox (B6.129-Atg5tm1Maday (RRBC02975)) mice were received from the RIKEN BioResource Center (BRC, Ibaraki, Japan). Conditional ATG5 KO mice were

**Figure 6 | Reduced neuronal complexity and neurodegeneration in the absence of neuronal AP-2m in vivo.** (a) Postnatal growth retardation of 21-day-old KO mice conditionally deleted for AP-2m by transgenic expression of Cre recombinase under the neuron-specific Tautulin promoter (AP-2lox/lox ×Tubulin1 Cre). See also Supplementary Figs 7a,b. (b) Kaplan–Meier survival curves of neuron-specific AP-2m KO mice and littermate controls (AP-2wt/wt ×Tubulin1 Cre (WT), AP-2lox/lox ×Tubulin1 Cre (Het) and AP-2lox/lox ×Tubulin1 Cre (KO)). (c,d) Golgi silver impregnation of cortices from p20 WT and AP-2m KO mice reveal the loss of dendritic architecture in AP-2m KO brain. Scale bars, 200 μm. (e,f) 3D morphology of stellate neurons in control (e) and AP-2m-deficient (f) brains. Scale bars, 40 μm. (g,h) Sholl analysis of stellate cells, revealing their branching complexity (g) and total dendritic length (h) in p20 WT and AP-2m KO brains. (i) Histopathological analysis of the brain of AP-2m KO mice at p20 shows marked degeneration of the thalamus (indicated by dotted line), but no overt alteration of the hippocampus (CA1). Nissl-stained brain sections of WT and conditional AP-2m KO mice, cc, corpus callosum; AV, anteroventral thalamic nucleus; AM, anteromedial thalamic nucleus; LD, laterodorsal thalamic nucleus; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VPL, ventral posterolateral thalamic nucleus, Re, reuniens thalamic nucleus; Rt, reticular nucleus. Scale bars, 800 μm. See also Supplementary Fig. 7g–p for an overview of the temporal progression of neurodegeneration in the brain of AP-2m KO mice. (j) Loss of barrel compartments in the somatosensory cortex of AP-2m KO mice. Cortical barrels visualized by immunostaining for potassium-chloride transporter member 2 are seen in WT controls, but absent in AP-2m KO brains. Roman numbers indicate cortical layers. Scale bars, 500 μm. (k,l) Representative confocal images of thalamic neurons in WT and AP-2m KO mice immunostained for p62. White rectangular boxes in k indicate areas magnified in l. Scale bars, 5 μm. (m) Mean size of p62-positive puncta is significantly increased in brains of AP-2m KO mice (1.92 ± 0.26) compared to WT littermates (1.26 ± 0.08, *P* = 0.041, *n* = 5). Data in m are illustrated as box plots as described in Methods. Data in b,g,h and all data reported in the text are mean ± s.e.m.
Figure 7 | Loss of neuronal complexity in AP-2μ-deficient neurons results from reduced TrkB signalling to control expression of BDNF.

(a) Hypothetical model for the role of AP-2 in retrograde transport of TrkB-containing autophagosomes in neurons. In WT neurons, AP-2 via its association with LC3 and p150Glued mediates retrograde transport of BDNF/TrkB-containing amphisomes (late-stage autophagosomes post-fusion with Rab7-positive late endosomes) to the cell body, where TrkB signalling regulates transcription of activity-dependent genes in the nucleus. In the absence of AP-2 (KO) TrkB endocytosis proceeds, however BDNF/TrkB-mediated signalling is defective due to impaired retrograde transport of BDNF/TrkB-containing autophagosomes. Stalled late-stage autophagosomes in neurites of AP-2 KO neurons cause axonal swellings and underlie neurodegeneration.

(b) Immunoblot analysis of BDNF expression levels in brain lysates from WT or conditional AP-2 KO mice. Note that AP-2μ remaining in the KO brains is largely derived from AP-2 expressed in glial cells not targeted by Cre. See also the levels of pro-BDNF in WT and AP-2 KO neurons in Supplementary Fig. 7v.

(c) BDNF levels are significantly decreased in AP-2 KO neurons (WT: 99.9 ± 0.02%, KO: 72.1 ± 1.12%, **P = 0.02). (d) BDNF mRNA levels are significantly decreased in AP-2 KO neurons (WT: 99.9 ± 0.02%, KO: 72.1 ± 1.12%, **P = 0.02). (e) Reduced neuronal complexity of AP-2μ-deficient neurons is rescued by long-term BDNF application. (e) AP-2μ KO neurons expressing eGFP were treated for 7 days with 50 ng ml⁻¹ BDNF or left untreated. Scale bars, 100 μm. (f) Application of BDNF rescues the number of branching points in neurons lacking AP-2μ compared to untreated controls (KO + BDNF: 66.65 ± 24.19, KO untreated: 34.87 ± 11.19, *P = 0.046). No difference in the number of branching points between untreated and BDNF-treated WT neurons was observed (WT + BDNF: 84.74 ± 21.49, WT untreated 80.86 ± 11.96, P = 0.882, n = 3 experiments, 21–23 neurons per condition). (g) NGF fails to rescue the neuronal complexity loss in AP-2μ-KO neurons (KO + NGF: 80.6 ± 4.2, KO untreated: 83.8 ± 4.2, P = 0.254; WT + NGF: 130.1 ± 6.3, WT untreated 137.6 ± 25.4, P = 0.774, n = 3 experiments). NS, non-significant. Data in (f,g) are illustrated as box plots as described in Methods. Data in (c,d) and all data in the text are mean ± s.e.m.

Plasmids. Expression plasmids encoding HA-tagged AP-2x WT or LC3-binding defective mutant AP-2Δ were a generous gift from Dr P. Greengard (Rockefeller University, New York, USA). cMyc-LC3a was generated from cMyc-LC3a, generously provided by Dr F. Reggiori (University of Utrecht, the Netherlands). mRFP-TrkB was a kind gift from Dr F. Saudou (France Grenoble Institut des Neurosciences, Grenoble, France). eGFP-TrkB was constructed by placing the coding sequence of TrkB lacking its own signal peptide downstream of the ApoER signal peptide in frame with eGFP. pSUPER (ref. 61), pEGFP-C2-BIO, HA-BirA (ref. 62) plasmids were kindly provided by Dr C.C. Hoogenraad (University of Utrecht, the Netherlands). β-actin-GFP and pEGFP-BIO-β-gal (AviGFP-β-gal) were described previously64. pEGFP-BIO-p150Glued (AviGFP-p150Glued) was obtained by cloning rat p150Glued cDNA, obtained by PCR using rat cDNA library, into pEGFP-C2-BIO. p150Glued shRNA sequence (5′-gatcgagagacagtcatca-3′) was designed against rat p150Glued mRNA and cloned.
introducing the full-length cDNA encoding mouse into pSuper vector. AP2mu-IRES-mRFP-in-AAV-HBA-EWB was generated by fusion PCR was inserted into the resulting construct via Age1/Bsp1407I restriction sites. Plasmid encoding GST-tagged human LC3B was a gift from Dr Volker Dotsch (University of Frankfurt, Germany). GST-tagged human STT2 (amino acids 1–555) has been described before. mCherry-ATG12 was a generous gift of Dr Michaelammers (CECAD, University of Cologne). Mito-mCherry was a kind gift of Dr Elena Rugarli (CECAD, University of Cologne), Munc-13-1-eYFP was a generous gift of Dr Neil Brose (Max Planck for Exp. Medicine, Göttingen, Germany).

Preparation of neuronal cell cultures and transfection. Neurons from cortex and hippocampus were isolated from postnatal mice at p1–5 as previously described. Cells were transfected at 7–9 days in vitro (DIV) using an optimized calcium phosphate protocol. To initiate homologous recombination in neurons from floxed animals expressing a tamoxifen-inducible Cre recombinase cultured neurons were treated with 0.25 μM (Z)-4-hydroxytamoxifen (Sigma) immediately after plating (DIV0). Equal concentrations of tamoxifen (0.25 μM) were used during medium renewal on DIV1 and DIV2. Ethanolic tamoxifen was added to control neurons in an amount equal to the tamoxifen concentration (0.25 μM). In some cases, homologous recombination was induced at a later stage by treating cultured neurons from floxed animals expressing a tamoxifen-inducible Cre recombinase with 0.25 μM (Z)-4-hydroxytamoxifen (Sigma) at DIV8. For rescue experiments homologous recombination was initiated by applying the tamoxifen at DIV20. AP2mu-IRES-mRFP construct was introduced at DIV8 and the neurons were analysed at DIV14.

Immunocytochemistry and analysis of cultured neurons. Neurons were fixed on DIV 13–16 in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min at RT, washed and permeabilized with 0.3% Triton X-100 (TX) for 15 min at room temperature. After blocking with PBS containing 10% normal goat serum (NGS), neurons were incubated with primary antibodies (see Supplementary Table 1) for 1 h in PBS containing 10% NGS and 0.3% Tx. Coverslips were rinsed three times with PBS (10 min each) and incubated with corresponding secondary antibodies (see Supplementary Table 1) for 30 min (diluted 1:400 in PBS containing 0.3% Tx and 10% NGS). Subsequently, coverslips were washed three times in PBS and mounted in ImmunoMount. For detection of LC3B and Rab7 permeabilization and blocking were performed in 5% BSA and 0.3% Saponin. The same buffer was used for antibody dilution.

Neurons were imaged at DIV 13–16 at a resolution of 1,024 × 1,024, with eight-bit sampling (no z increment was used) with both chromatic and spherical aberrations. Pearson's correlation coefficients (Rp) were determined in ROIs (11.7 × 13.3 μm) from non-processed raw dual channel images using the Intensity Correlation Analysis function from the Colocalization Macro in ImageJ (ref. 67). For quantitative analysis of fluorescent puncta the total area of the neuron was manually selected using ImageJ selection tools. Fluorescent puncta were determined by applying the autothreshold ‘minimum’ algorithm implemented in ImageJ and analysed using the ‘Analyse particles’ ImageJ module to determine the number of fluorescent puncta per μm². All antibodies used for immunostaining are indicated in Supplementary Table 1 (that is, IF).

**STED imaging and analysis.** STED imaging with time-gated detection was performed using a commercial Leica Sp5 TCS STED microscope (Leica Microsystems) equipped with a pulsed excitation white light laser (WLL; ~80-ps pulse width, 80-MHz repetition rate; NKT Photonics) and two STED lasers for depletion (continuous wave at 925 nm, pulsed at 775 nm). The pulsed 775-nm STED laser was triggered by the WLL. Within each independent experiment, samples were acquired with equal settings. Alexa 488 and Alexa 555 were excited using a pulsed WLL at 488 and 545 nm, respectively. Depletion occurred at 592 nm. Fluorescence signals were detected sequentially from the STED laser by corresponding dichroic filters. Images were acquired with an HC PL APO CS2 100/1.40-N.A. oil objective (Leica Microsystems), a scanning format of 1,024, eight-bit sampling, and 1,024, eight-bit sampling, respectively. Depletion (continuous wave at 592 nm, pulsed at 775 nm). 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HeLa cell siRNA and plasmid transfections. HeLa cells were seeded to two rounds of transfection (days 0 and 2) with siRNA using Oligofectamine (Life Technologies) according to the manufacturer’s protocol. For additional transient overexpression, plasmids were transfected on day 4 using X-tremeGENE 9 DNA transfection reagent (Roche). For silencing, the following siRNA was used: AP-2β 5'-AAGAACUGUGACGUCAGCUCGG-3' Scrambled AP-2α sequence 5'-GTAAC TCGCGGCTCGTGGTTT-3' was used as control siRNA.

Transferrin uptake in HeLa cells. HeLa cells seeded on coverslips coated with Matrigel (BD Biosciences) were serum-starved for 1 h and treated with 25 μg ml⁻¹ Tf-Alexa647 (Life Technologies) for 10 min at 37°C. Cells were washed twice with ice-cold PBS and acid washed at pH 5.3 (0.1 M Na-acetate, 0.2 M NaCl) for 1 min on ice. The coverslips were washed twice with ice-cold PBS and fixed with 4% PFA for 30 min at room temperature. Transferrin uptake was analysed using a Nikon Eclipse Ti microscope equipped with a × 40 oil-immersion objective and quantified using ImageJ software.

Immunoblot analysis of mouse brain extracts. Somatosensory cortices were homogenized in lysis buffer (20 mM Heps-KOH, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 1% Triton X-100, supplemented with 1 mM PMSF and mammalian protease and phosphatase inhibitor mixture) using a glass-fenlon homogenizer. The lysate was incubated 20 min on ice before centrifugation at 17,000g for 10 min at 4°C. The protein concentration was determined by Bradford assay. Samples were analysed by SDS–PAGE and immunoblotting. Antibodies were detected using HRP-coupled secondary antibodies in a chemo-luminescence reaction or with the Odyssey LiCor system. All antibodies used for immunoblotting are indicated in Supplementary Table 1 (under WB). Images in Fig 7b, supplementary Figs S1, 4g, h, 5g, l, 6f, 7e have been cropped for presentation. Full size images are presented in supplementary Fig. 8.

Immunoprecipitation. Dynabeads Protein G (50 μl) were washed in lysis buffer (150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM Heps-NaOH, pH 7.5) supplemented with 0.3% CHAPS and protease, as well as phosphatase inhibitors. Beads were washed twice with lysis buffer and fixed with 4% PFA for 30 min at room temperature. Transferrin uptake was analysed using a Nikon Eclipse Ti microscope equipped with a × 40 oil-immersion objective and quantified using ImageJ software.

Binding of biotinylated p150phox to the J2-ear. M-280 streptavidin Dynabeads were blocked for 1 h in 20 mM Heps-KOH, pH 7.5, 150 mM KCl supplemented with 0.2 mM BSA and 20% glycerol. Beads were washed with lysis buffer (20 mM Heps-KOH, pH 7.5, 150 mM KCl, 0.1% CHAPS) supplemented with protease- and phosphatase inhibitors. HEK293T cells co-expressing AviGFP-p150phox or AviGFP-f-galactosidase as a negative control were lysed for 15 min on ice in lysis buffer. The supernatant was added to the prepared M-280 streptavidin Dynabeads for 1 h at 4°C while rotating. Beads were then washed four times in washing buffer (20 mM Heps-KOH, pH 7.5, 150 mM KCl, 0.1% CHAPS) and incubated with 0.2 mg ml⁻¹ His₄-AP-2β appendage domain in binding buffer: 20 mM Heps-KOH pH 7.5, 300 mM KCl and 0.1% CHAPS for 2 h at 4°C rotating. Finally, beads were washed four times with binding buffer, eluted in SDS sample buffer and analysed via SDS–PAGE and subsequent immunoblotting. Images in Fig 2d have been cropped for presentation. Full size images are presented in Supplementary Fig. 8.

Brain lysate pull-down assay. GST- and His-tagged fusion proteins were affinity-purified by glutathione-sepharose respectively Ni-NTA based affinity chromatography. GST-fusion-proteins were stored at 4°C in PBS. Detergent extracted mouse brain lysates were prepared as described. Briefly, mouse brains were homogenized in lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM DTT, 0.005% Triton X-100, supplemented with protease- and phosphatase inhibitors) and incubated on ice for 30 min before centrifugation at 65,000 x r.p.m. for 15 min at 4°C. Supernatant was added to 50 μg recombinant purified GST or GST-AP-2β and rotated for 2 h at 4°C. Samples were washed five times with lysis buffer, eluted in SDS sample buffer and analysed via SDS–PAGE and immunoblotting. Images in Fig 2e and Supplementary Fig. 2e have been cropped for presentation. Full size images are presented in Supplementary Fig. 8.

Quantitative real-time RT – PCR (qRT-PCR). Total RNA from cultured ethanol or tamoxifen-treated neurons from AP-2β/CAG-Cre mice was extracted using TRIzol reagent (ambion, RNA, Life Technologies). After homogenizing the samples with TRIzol reagent, chloroform was added and the aqueous layer containing the RNA was isolated and precipitated with isopropanol. Reverse transcription into cDNA was carried out by using the High Capacity cDNA Reverse Transcription Kit (4368814, Thermo Scientific) according to manufacturers’ instructions. qRT-PCR was performed with a LightCycler 1.5 instrument using Fast Start DNA Master SYBR Green 1 Kit (Roche). Primer sequences are listed in Supplementary Table 2. Samples were run in duplicates and BDNF signals were normalized to GAPDH intensity.

Statistical analysis. For analysis of experiments, statistically significant estimates were obtained from independent experiments (n). The statistical significance between two groups for all normally distributed raw data except growth factor treatments was evaluated with a two-tailed unpaired student’s t-test. Effects of BDNF and NGF on neuronal complexity were evaluated using paired student’s t-tests. The statistical significance between more than two groups for all normally distributed raw data (Fig. 4b,d) was evaluated using one-way ANOVA (Tukey post-hoc test was used to determine the statistical significance between the groups). All normalized data (Figs 4b and 7c.d, Supplementary Figs S1, 4g, 5h, l) were evaluated using one-sample student’s t-test. Significant differences were accepted at P < 0.05. For box plots the median divides the box, while the upper boundary of the box corresponds to the third quartile and the lower boundary corresponds to the first quartile. The minimum and the maximum values extend as bars from the bottom and top of the box.

Antibodies. An overview of all antibodies used in this study is given in Supplementary Table 1.

Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request.

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Acknowledgements
We thank Sabine Hahn, Delia Löwe, Nina Ellrich and Silke Zillmann for expert technical assistance. Supported by grants from the German Research Foundation (SFB958/A01 to V.H. and T.M.), the NeuroCure Cluster of Excellence (Exc-257) and the Reinhart-Koselleck Award (to V.H.). Work by N.L.K. was supported by a NeuroCure Female PostDoc fellowship and financed by the Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD, Exc 229). Work of J.J., A.T., A.R.M., A.S. was financed by a National Science Centre grant no. 2011/03/B/NZ3/01970. J.J. and A.R.M. are also recipients of the Foundation for Polish Science ‘Mistra’ Professorial Subsidy and Fellowship, respectively. M.K. received funding from an H2020 EU Marie Curie IF fellowship. Furthermore, we thank Prof. Paul Greengard (Rockefeller University, New York, USA) for providing plasmids for WT and LC3-binding defective mutant AP-2a, Dr Fulvio Reggiori (University of Utrecht, Netherlands) for the Mgc-LC3 plasmid, Prof Volker Dotsch (University of Frankfurt, Germany) for the GST-LC3b construct, Dr F. Saudou (Grenoble Grenoble Institut des Neurosciences, Grenoble, France) for the TrkB-mRFP plasmid, Dr N. Brose (Max Planck for Experimental Medicine, Göttingen, Germany) for the Munc-13-1-eYFP plasmid, Dr Edela Rugarli (CECAD, University of Cologne) for the Mito-mCherry plasmid and Dr Lammers (CECAD, University of Cologne, Germany) for the mCherry-ATG12 plasmid. We are grateful to Dr Min Kye for the generous help with qPCR experiments.

We appreciate the help of the CECAD Imaging Facility, especially Dr Christian Jüngst for the assistance in scanning histology slides.

Author contributions
N.L.K., G.A.C., M.K., D.P., A.R.M., S.B., A.T., A.S. and J.J. performed experiments, T.M. contributed reagents, N.L.K., J.J. and V.H., designed research. N.L.K. and V.H. wrote the manuscript with input from all authors.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing interests: The authors declare no competing financial interests.

How to cite this article: Kononenko, N. L. et al. Retrograde transport of TrkB-containing autophagosomes via the adaptor AP-2 mediates neuronal complexity and prevents neurodegeneration. Nat. Commun. 8, 14819 doi: 10.1038/ncomms14819 (2017).

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