The GTPase Arl8B Plays a Principle Role in the Positioning of Interstitial Axon Branches by Spatially Controlling Autophagosome and Lysosome Location

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Interstitial axon branching is an essential step during the establishment of neuronal connectivity. However, the exact mechanisms on how the number and position of branches are determined are still not fully understood. Here, we investigated the role of Arl8B, an adaptor molecule between lysosomes and kinesins. In chick retinal ganglion cells (RGCs), downregulation of Arl8B reduces axon branch density and shifts their location more proximally, while Arl8B overexpression leads to increased density and more distal positions of branches. These alterations correlate with changes in the location and density of lysosomes and autophagosomes along the axon shaft. Diminishing autophagy directly by knock-down of atg7, a key autophagy gene, reduces branch density, while induction of autophagy by rapamycin increases axon branching, indicating that autophagy plays a prominent role in axon branch formation. In vivo, local inactivation of autophagy in the retina using a mouse conditional knock-out approach disturbs retino-collicular map formation which is dependent on the formation of interstitial axon branches. These data suggest that Arl8B plays a principal role in the positioning of axon branches by spatially controlling autophagy, thus directly controlling formation of neural connectivity in the brain.

Key words: autophagy; axon branching; lysosomes; neural circuit development; retinotectal projection; vesicle trafficking

Significance Statement

The formation of interstitial axonal branches plays a prominent role in numerous places of the developing brain during neural circuit establishment. We show here that the GTPase Arl8B controls density and location of interstitial axon branches, and at the same time controls also density and location of the autophagy machinery. Upregulation or downregulation of autophagy in vitro promotes or inhibits axon branching. Local disruption of autophagy in vivo disturbs retino-collicular mapping. Our data suggest that Arl8B controls axon branching by controlling locally autophagy. This work is one of the first reports showing a role of autophagy during early neural circuit development and suggests that autophagy in general plays a much more prominent role during brain development than previously anticipated.

Introduction

Axonal branches, which extend interstitially from axon shafts and arborize at specific target locations, are responsible for most, if not all, neural connectivity in the vertebrate CNS (Gallo, 2011; Kalil and Dent, 2014).

The formation of interstitial branches requires cytoskeletal rearrangements at branch points. Here, actin assembly often initiates filopodium formation followed by microtubule (MT) invasion which marks the maturation of the interstitial branches. Various processes have been implicated in controlling these cytoskeletal rearrangements. These include local signaling by membrane-bound receptors (e.g., EphA/ephrinAs; BDNF/TrkB; Yates et al., 2001; Marler et al., 2008), local transport and translation of specific RNAs to sites of emerging branches (Wong et al., 2017; Cioni et al., 2019), and the capture of mitochondria (Courchet et al., 2013; Lewis et al., 2018).

In Caenorhabditis elegans, local assembly of an F-actin network initiates synapse formation and axon branching in parallel...
(Chia et al., 2014). Local clustering of synaptic vesicles (SVs) can trigger the development of axonal branches and arborization, as described in the synaptotrophic hypothesis (Vaughn, 1989; Alsina et al., 2001; Meyer and Smith, 2006; Ruthazer et al., 2006; Cline and Haas, 2008; Matsumoto et al., 2016; Constance et al., 2018). It is not clear though which factors determine where presynaptic clusters are formed. In C. elegans, the small GTPase Arl8 has been shown to determine the position and number of presynaptic sites along axons of DA9 neurons (Klassen et al., 2010; Wu et al., 2013). Arl8 is also involved in the maintenance of presynaptic structures in Drosophila, mediating axonal cotransport of active zone (AZ) proteins and SV proteins in lysosome-related vesicles (Vukoja et al., 2018; Goel et al., 2019).

Arl8 belongs to the Ras superfamily of small GTP-binding proteins that switch between a membrane- and GTP-bound active form, and a cytosolic, inactive GDP-bound form (Gillingham and Munro, 2007). While members of the extended families of Arf and Arf-like (Arls) molecules participate in various aspects of membrane organelle traffic, Arl8 is the only G protein localized specifically on lysosomes (Bagshaw et al., 2006; Garg et al., 2011; Korolchuk et al., 2011; Rosa-Ferreira and Munro, 2011; Khatter et al., 2015b).

Lysosomes are a central hub of cellular homeostasis. They degrade and recycle cell-intrinsic structures and extracellular material (Harris and Rubinsztein, 2011; Hurley and Schulman, 2014; Feng et al., 2015), and control metabolic signaling, plasma membrane repair, exocytosis, cell adhesion and migration (Pu et al., 2016). The subcellular location of lysosomes is of crucial importance for their activity (Wong and Cuervo, 2010; Korolchuk et al., 2011; Galluzzi et al., 2014; Hurley and Schulman, 2014; Pu et al., 2016; Bonifácino and Neefjes, 2017; Ferguson, 2018). For example, engulfment of cytoplasmic proteins and organelles by autophagosomes attracts lysosomes and enables their fusion to autolysosomes and the later degradation of engulfed material (Maday et al., 2012; Bento et al., 2013; Maday and Holzbaur, 2014; Filipek et al., 2017; Pu et al., 2017).

Arl8B has been identified as a key regulator of lysosome movement (for review, see Khatter et al., 2015a), functioning as an adaptor molecule linking lysosomes to MT-bound kinesin motor proteins to mediate an MT plus-end directed transport (Hofmann and Munro, 2006; Korolchuk et al., 2011; Pu et al., 2015; Niwa et al., 2016, 2017; Farias et al., 2017).

Arl8B regulates not only the transport of lysosomes into axons (Farias et al., 2017; Rosa-Ferreira et al., 2018) but is also involved in their fusion with autophagosomes and late endosomes by recruiting the homotypic fusion and protein sorting (HOPS) complex (Khattet al., 2015b; McEwan et al., 2015; Marwaha et al., 2017; Boda et al., 2019).

Here, we show that Arl8B controls the positioning and density of interstitial branching of retinal ganglion cell (RGC) axons, and at the same time controls also positioning and density of lysosomes and autophagosomes. This suggests that Arl8B plays a major role in defining the earliest stages of axon branch formation by locally controlling the dynamics of vesicles involved in autophagy.

Materials and Methods

Cloning of Arl8B expression constructs

Chicken Arl8B cDNA was generated using primers 5′–GCTTACGTTCCACGCCTCTT–3′ and 3′–ACCCAGGAGAATGTCCTGGTCTGC–3′ from reverse transcribed chick retinal RNA as a template. This cDNA was cloned into expression vectors containing either tdTomato or GFP fluorescent proteins. Here, expression of the fusion proteins (NH2–Arl8B–tdTomato/GFP–COOH) is under the control of the CMV and β–actin (CAG) promoter. The LAMP–YFP and the fluorescent-LC3 plasmids were purchased from Addgene (plasmid no. 1816) and a gift of Terje Johansen (Tromso, Norway).

Mouse Arl8B cDNA was cloned from mouse brain RNA using RT–PCR into the same vectors with forward primer 5′–GGCTTCTCCGGGAATTGAGGTTGCTAA–3′ and reverse primer 5′–GGCTTCTCCGGGATTGGAGGTTGCTAA–3′. All expression constructs generated by RT–PCR were sequence verified.

Primary chick neuronal cultures

Primary chick retinal cultures were prepared as described in Marler et al. (2008). In brief, nasal retinas were isolated from embryonic day (E7) or E8 chicken embryos, dissociated to obtain a single-cell suspension and electroporated using an Amaxa Nucleofector (Lonza). Neuronal cultures were plated on poly-L-lysine (Sigma), laminin (Life Technologies)-coated and mersine (Millipore)-coated surfaces and grown for 2–3 d at 37°C in Neurobasal medium (Life Technologies), supplemented with B27 (Life Technologies), L-glutamine (Invitrogen), and forskolin (Sigma) in presence of penicillin/streptomycin (Life Technologies).

Antibody staining

After 2–3 d in culture, retinal neuronal cultures were fixed using 4% paraformaldehyde (PFA), 30% sucrose in PBS, and stained for endogenous expression of Arl8B using a rabbit anti-Arl8B antibody (13049-1-AP from Proteintech), which was raised by immunization with a human Arl8B fusion protein.

In brief, after PFA fixation, cells were three times washed with 1 × PBS, 0.1% Triton X-100 (PBST). For antigen retrieval, cells were incubated for 1 h at 60°C in 0.1% citrate buffer, pH 6.0, and washed four times in PBST, blocked in 5% BSA/10% donkey serum/0.1% Triton X-100 (Blocking buffer-1) for 2 h min at room temperature (RT), incubated with first antibody (anti-Arl8B diluted 1:100; anti-β tubulin 1:500) in Blocking buffer-2 (1% BSA; 5% donkey serum; 0.1% Triton X-100; for specificity control in Blocking buffer-2 only) at 4°C overnight, washed four times in PBST, incubated with anti-rabbit Alexa Fluor 488 antibody (1:1000); anti‐mouse Alexa Fluor 647 antibody in Blocking buffer-2 for 2 h at RT, washed four times in PBST, and stored under Mowiol. Analyses were done using a Zeiss confocal LSM800.

LAMPI was stained using rabbit anti-LAMPI antibody (Abcam ab24170). Neurons were counterstained with DAPI (Roche) to visualize cell nuclei.

Arl8B knock-down analysis

For Arl8B knock-down experiments, a series of short hairpin RNAs (shRNAs), expressed from the U6 promoter, were analyzed. These plasmids contained in addition a TagRFP expression cassette allowing the subsequent identification of electroporated axons. To select (the most) efficient shRNAs, these constructs were transfected together with an Arl8B-GFP expression plasmid into Chinese hamster ovary (CHO) cells or primary chick retinal neurons. After 2 d in culture, Arl8B expression levels were determined by Western blot analysis using an anti-GFP antibody (Clontech 632496). Anti-GAPDH antibody was used to normalize Arl8B-GFP band intensities. Western blotting results were quantified using ImageJ and Excel software.

The shRNA showing the strongest downregulation [relative to control (scrambled) shRNAs] was used for further functional characterizations. This shRNA targeted the Arl8B mRNA between nucleotides 322 and 344. ShRNAs that do not target any known mRNA (“scrambled shRNAs”) were used as a control in knock-down experiments.

Atg7 knock-down analysis

For ATG7 knock-down experiments, the RNA of chicken Atg7 was targeted using the mirRNA- based shRNA silencing method (Shan et al., 2009). Several shRNA inserts were assembled by PCR using a series of primers including: FOR1 5′–TGCTTCTGGCCTGTCATGTT–3′; REV1 5′–AGTAACAGGCACTCATACACTGAAGTCCTGACCAATATGAACTGACCTGACCT–3′; and 3′–reverse primer 5′–GGCTTCTCCGGGAATTGAGGTTGCTAA–3′. All expression constructs generated by RT–PCR were sequence verified.
GTCAAGGGCACCAAGC −3′, followed by FOR2 5′−ATTGATCGT
ATTGAGGCTGTGTTAGGCTGTGTCG−3′ and REV2 5′−GC
AAATTTCCAGCAATTGGTTCTATTGTAATGACAGGC
ATCATACAGCT−3′. The resulting 154-nucleotide fragment was cleaved at Clal and EcoRI restriction sites and cloned into an eGFP vector, under
the control of the CAG promoter (Furukawa et al., 2011). The silencing ef-
ciency was assessed by transfecting various constructs into CHO cells to-
gether with an Atg7-Flag-expressing vector. After 3 d in culture, Atg7
expression levels were analyzed by Western blotting using an anti-FLAG
antibody (Sigma F1804). As a control, a shRNA plasmid containing sequences that do not target any known mRNA (shRNA) was used. The shRNA with the strongest knock-down eficiency (sequence shown above) was used for additional experiments.

Chicken Atg7 was ampliﬁed from reverse transcribed chick retinal RNA as a template using primers 5′−AAGCTTCAAGTGGCG
GCGACTGGAATG−3′ and 5′−CTCGAGACGTGGCATC
ACTCAGTGTC−3′, 205 cleaved at HindIII and XhoI restriction sites and cloned into the pCMV-3 Tag-3 vector (Agilent).

Experimental design and statistical analyses
The experimental design and statistical analyses are described in detail for every single ﬁgure and experiment.

Axon branching assay
To analyze the consequences of upregulation or knock-down of Arl8B, or of Atg7, on axon branching, E7 or E8 single-cell retinal cultures were elec-
ropropated with either an Arl8B-GFP expression plasmid, Arl8B (or Atg7) shRNA plasmids, GFP-RFP-LC3 (Jain et al., 2010) or control con-
structs. Axon branching was analyzed after 3 d in culture.

Generally, for functional knock-down/overexpression experiments, 5−10 μg of DNA was electroporated per single-cell retinal prep, for experiments to visualize particular structures (lysosomes, LC3-II puncta, etc.) including colocalization experiments, only 0.5 μg of DNA per single-
cell retinal prep was electroporated.
A total of 5 ng/ml BDNF was added 24 h after plating (Marler et al., 2008). After 3 d in vitro, retinal cultures were ﬁxed and imaged by ﬂuo-
rescent microscopy, and axon length and branch number were analyzed using MetaMorph, ImageJ (using the Neuron) plug-in, Microsoft Excel,
and GraphPad Prism software. RGCs were identiﬁed based on their neu-
rone morphology and by immunocytochemical staining using RGC-spe-
ciﬁc antibodies as described previously (Marler et al., 2008).

Only branches longer than 5 μm were considered (Marler et al., 2008; Matsumoto et al., 2016). Branch density was determined as the
number of axon branches per unit of axon length. Branch density is
given as mean percentage values (normalized against the appropriate
control neurons) ± SEM.

All analyses were done blind to the condition. Statistical signiﬁcance between the conditions was tested using Student’s t test.

Analysis of particle distribution
RGC axons expressing fusion proteins (GFP, RFP, tdTomato, mCherry)
were observed using a confocal Zeiss LSM800 with a 63× objective lens. Tiles were used to capture entire RGCs and stitched using the Carl Zeiss
Microscopy software Zen 2.3 (blue edition). Z-stacks captured axonal puncta in their focal plane and were collapsed using the ImageJ tool
“Z Project.”

ImageJ tools were used to analyze the proximo-distal distribution of
lysosomes, autophagosomes, or SV clusters along RGC axons in control,
Arl8B KD, Atg7 KD and other approaches (baﬁlomycin, rapamycin).
The segmented line tool was used to trace the axon of each RGC, and the proﬁle of pixel intensities was plotted. A background value, deﬁned as the minimum signal value, was subtracted uniformly from each axon.

The sum of pixel intensities of the proximal, middle, and distal one-third compartments was individually calculated as a percentage of the sum of pixel intensities across the axon. The mean of each one-third compartment was calculated per condition. Statistical signiﬁcance between con-
ditions was tested using Student’s t test.

Quantification of puncta in axons, colocalization analysis (using colo2)
The Fiji plugin ‘straighten’ is used to straighten RGC axons to exclude extracellular noise or irrelevant somatic signals. The amount of colocali-
Zation of two ﬂuorescent-labeled proteins in the axon is automatically
quantified using the ImageJ plugin ‘Colo2’ (Cioni et al., 2018; Ordonez et al., 2018; Bonanomi et al., 2019). This performs pixel intensity analysis of correlation between the two channels in the straightened axon using Pearson’s linear correlation analysis, which gives a correlation coefﬁcient value r. As a control for random overlap, Colo2 shufﬂes pixel groups of one channel and repeats the correlation analysis against the second channel, giving a second r value. Colo2 also uses the Costes method to test for statistical signiﬁcance between the r values (p > 0.05 signify a statistical signiﬁcance). The mean r value for each condition is calculated using Fisher z-transformation.

Quantification of puncta in axons, distribution of low-density puncta
For puncta that naturally occur in very low densities in the axon (such as LC3), a different method was used to quantify their distribution and
density with greater accuracy. A series of ImageJ tools were used to quantify puncta density and distribution in the proximo-distal axis of
individual axons. All axons were initially traced and straightened using the ImageJ tools “Segmented Line” and “Straighten,” respectively.
Puncta along each axon were identiﬁed using similar thresholding meth-
ods to the established Otsu method (Otsu, 1979; Matsumoto et al.,
2016), namely “intermodes,” which is a histogram-derived thresholding
method that operates iterative smoothing until two local maxima remain. This method best identiﬁed individual puncta in proximity with each other. The plugin “Particle Analyzer” was used to quantify particles above threshold. Particles were only identiﬁed as puncta if they were larger than two pixels (~0.1 μm).

Particle analyzer also allows the proximo-distal coordinates of puncta in a straightened axon to be measured. Each axon was then divided into three equal segments (proximal, middle, and distal thirds) and the coor-
dinates of each punctum was used to quantify the number of puncta in
each axon segments. The mean proportion of puncta in each axon seg-
ment was then calculated as a percentage for each condition. To calculate relative puncta density, the total number of puncta was divided by the
axon length to give puncta density. Puncta densities were normalized to the mean puncta density in controls to give relative puncta density for
each axon.

Quantification of density of Dil-labeled termination zones (TZs)
Whole superior colliculi (SC) of pax6–/cre; atg2fl/fl mice and atg7fl/fl mice; cre mice were carefully dissected out and immediately imaged using a Zeiss AxioScope ﬂuorescence microscope with a 568 nm excitation
argon laser. A 5 × objective was used to capture entire SC and any Dil-la-
beled TZs.

Images were processed and TZs analyzed using ImageJ tools as fol-
ows. SCs in all images were outlined as region of interest (ROI) excluding
all other signals. TZs were identiﬁed using the tool thresholding method “Triangle,” a geometric method which assumes a maximum
peak in the histogram and best identiﬁes weak TZ signals in the SC. Any
background noise was identiﬁed using the remaining area of the SC
which does not contain a TZ. The density of TZs was ﬁnally calculated as
the raw integral values (R) minus background noise (B) divided by the
area of the TZ (A), which previously was determined by thresholding
(TZ density = (R − B)/A).

The mean TZ density was calculated for each condition and signiﬁ-
cant difference was determined using an independent-samples t test.

Results
Arl8B colocalizes with lysosomes in the axonal compartment
Interstitial axon branching is a critical step during map development
in the retino-tectal projection (Yates et al., 2001; Hindges et al., 2002; Marler et al., 2014). Here, RGC axons initially grow into the tectum in a non-topographic manner and “overshoot” their future TZs substantially. Topographically speciﬁc intersti-
tial branching subsequently leads to the formation of TZs, and the
development of a topographic map. Overshoot axon segments
are later removed. Members of the EphA/ephrinA family in concert with neurotrophic factors, such as BDNF and NT3, play prominent roles in this branching process (for review, see Suetterlin et al., 2012; Cang and Feldheim, 2013; Weth et al., 2014).

To characterize possible roles of Arl8B in this process, the first step was to determine its subcellular localization along RGC axons. We stained retinal cultures from E8 chicks after 2 d in culture with an Arl8B-specific antibody (see Materials and Methods). This time point corresponds to the major phase of retino-tectal map development between E10 and E14 during which RGC axons form TZs in the tectum through intense interstitial axon branching and subsequent arborization. Our data show a punctate expression pattern throughout the RGC axonal compartment (Fig. 1A, B).

As Arl8B is bound specifically to lysosomal and late endosomal membranes in vertebrate neuronal and non-neuronal cells...
We investigated whether this is also true for RGC axons. We expressed an Arl8B-tdTomato fusion protein in E8 single-cell retinal preparations and stained these cultures 3 d later for LAMP1, a specific marker for lysosomes. RGCs were identified based on their neurite morphology and by immunocytochemical staining using RGC-specific antibodies (as previously; Marler et al., 2008). Super-resolution structured illumination microscopy (SIM) showed an extensive colocalization of fluorescently labeled Arl8B with LAMP1 puncta (Fig. 1C–G) confirming that in RGC axons Arl8B associates with lysosomes. Approximately 80% of the LAMP1 puncta were positive for Arl8B, while a small fraction of Arl8B-positive puncta was negative for LAMP1 (Fig. 1F,G).

These findings are consistent with recent data from mouse hippocampal neurons (Farias et al., 2017; Vukoja et al., 2018).

A further developmental analysis showed that Arl8B protein, identified by specific antibodies, is localized in axons not only at the time of RGC branching within the tectum (Fig. 1A, I), but already at E6, a time when RGC axons have just reached this area (Fig. 1H), indicating a role of Arl8B beyond axon branching.

Figure 2. Arl8B controls RGC axon branch density and position. E8 chick single retinal cells were electroporated with expression constructs to either knock-down or to overexpress Arl8B. After 3 d in culture, neurons were fixed, and RGC axon length and branch number determined. Branch density is given as the number of axon branches per unit axon length. The values were normalized against cultures expressing corresponding control constructs. Axon length and branch number were analyzed using MetaMorph and NeuronJ. For each experimental condition, three independent experiments were performed with n > 15 axons for each experiment. A–D, After knock-down or overexpression of Arl8B, the density of RGC axon branches was determined. A–C, Representative images of RGC axons from (A) control, (B) Arl8B overexpression, and (C) Arl8B shRNA knock-down. D, Quantification of branch counts in the different conditions. Arl8B overexpression increases the number of axon branches by a factor of ~2 (to 201.1 ± 23.7%; control 100%), while Arl8B knock-down decreased axon branching by about two-thirds to 37.6 ± 3.8%. The characterization of Arl8B shRNA including rescue experiments are shown in Figure 3. Scale bar in A–C: 5 μm. E–I, Determination of the proximo-distal location of RGC axon branches after knock-down or overexpression of Arl8B. E–G, Representative images of RGC axons from control cultures (E), Arl8B overexpression (F), and Arl8B shRNA knock-down (G). H, Arl8B overexpression decreased axon branching to 59.8 ± 5.4% in the proximal third, while (I) Arl8B shRNA knock-down increased the fraction of axon branches in the proximal third to 169.4 ± 9.4% (control = 100 ± 9%). Statistical analysis was performed using Student’s t test. Error bars denote SEM, and significance is indicated as ***p < 0.001 and *p < 0.05. Scale bar in E–G: 5 μm. J, Schematic summary of the results from overexpression and knock-down of Arl8B on RGC branch location and number.

Arl8B controls the density of axon branches
To test for a role of Arl8B in controlling interstitial axon branching, we employed both an overexpression and a knock-down approach using shRNAs (Fig. 2).
In the gain of function approach, we analyzed the effects of Arl8B over-expression on axon morphology. We used here an Arl8B-GFP fusion protein which has been shown previously to be functionally intact (Korolchuk et al., 2011). Overexpression of Arl8B-GFP in cultured chick RGCs resulted in a ~2-fold increase in axon branch density [201.1 ± 23.7% (mean ± SEM)] compared with cultures transfected with control plasmid (Fig. 2A,B,D).

For the complementary knock-down approach, primary cultures from E8 chick retina were electroporated with either Arl8B shRNA plasmids (described in Fig. 3) or control shRNA constructs and analyzed by fluorescent microscopy after 3 d in culture. Knock-down of Arl8B resulted in a decrease in interstitial axon branch density (number of axonal branches per unit of axon length) to 37.6 ± 3.8% (mean ± SEM) of branch density in control transfected cultures (= 100%; Fig. 2A,C,D). Arl8B knock-down had no significant effect on axon length (96.34 ± 4.8 mm for Arl8B shRNA vs 93.17 ± 4.0 mm for control shRNA).

Taken together, these results show that the expression level of Arl8B controls axon branch density in cultured RGCs.

**Arl8B expression level affects the location of axon branches**

A crucial aspect for the development of the topographically specific formation of TZs during retino-tectal map development is where along the axon branches are formed. We therefore analyzed next whether changing Arl8B expression levels affect also the proximo-distal positioning of branches along RGC axons (Fig. 2E–I). We found that overexpression of Arl8B decreased the fraction of branches in the proximal third of axons (Fig. 2F, H), while a knock-down of Arl8B increases the fraction of branches formed in this area, compared with controls (Fig. 2G). Thus, after Arl8B knockdown, the fraction of branches in the proximal third rose to 169.4 ± 9.4% (mean ± SEM) compared with controls (100%), while upregulation reduced the fraction of branches to 59.8 ± 5.4% (mean ± SEM) of controls (Fig. 2H,I).

Our results therefore show that Arl8B controls the density and position of RGC axon branches, as schematized in Figure 2J.

**Characterization of Arl8B shRNAs**

To confirm their function in knocking down Arl8B, the shRNAs used were transfected together with an Arl8B-GFP expression plasmid into CHO cells or primary chick retinal neurons.
Figure 4. Modulation of Arl8B expression affects density and position of lysosomes in RGC axons. A–D, Retinal cultures were transfected with expression plasmids for Arl8B-GFP (A, B, B’) or GFP (C, D, D’) and small amounts of LAMP1-RFP (E–G) to visualize lysosome distributions. After 3 d in culture, neurons were analyzed for LAMP1-RFP fluorescence intensity and distribution (E–G). The sum of three independent experiments is shown. B’, D’. Higher magnifications of axonal segments from B, D, respectively, to illustrate the increase in lysosome density in the distal axon after Arl8B-GFP overexpression. Overexpression of Arl8B-GFP results in a significant increase in the intensity of LAMP1-RFP (E). Axon length is not affected (F). Lysosome-RFP intensity is decreased in the proximal third of axons, while it is increased in the distal third (G). H–L, Retinal cultures were transfected with control shRNA (H) or Arl8B shRNA (I), together with small amounts of LAMP1-GFP (J, I) to visualize lysosome distributions. Cultures were fixed 3 d later and analyzed for LAMP1-GFP fluorescence distribution along the axons. A quantification of the LAMP1-GFP distribution is given in J–L. Arl8B knock-down results in a significant reduction in the overall intensity of LAMP1-GFP fluorescence (J), and a fluorescence shift to the proximal third of axons (L). Axon length is not affected (K). The quantification method used is described in Materials and Methods. Statistical analysis was performed using Student’s t test. Error bars denote SEM and significance is indicated as *p < 0.05 and ***p < 0.001; n.s., not significant. The number of axons analyzed (n) is given within the columns. Scale bars: 20 μm (A–D) and 10 μm (H, I, CB: cell body.

2 d in culture, Arl8B-GFP expression levels relative to control (scrambled) shRNA transfected cells were significantly downregulated (Fig. 3A–D).

We used branch density as a parameter to further investigate possible off-target effects of the Arl8B shRNA, i.e., its specificity in knocking down Arl8B (Fig. 3E–G). For this, we analyzed whether the reduced branch density mediated by the shRNA for chick Arl8B mRNA could be rescued by overexpression of mouse Arl8B protein. A sequence alignment showed a difference in four out of 20 nucleotides between the shRNA target region in the chick sequence, and the corresponding region in the mouse Arl8B sequence (Fig. 3G), making it unlikely that the chick shRNA binds to mouse Arl8B mRNA. We found that overexpression of mouse Arl8B rescues the shRNA-mediated down regulation of branching (Fig. 3E). We conclude from this that the effect of the chick shRNA is specific for Arl8B and unlikely because of an off-target effect. As before, axon length was not affected by these manipulations (Fig. 3F).

Further proof for the specificity of the Arl8B shRNA was obtained by staining axons for endogenous Arl8B protein (see Materials and Methods) after shRNA transfection. We found a strong and statistically significant reduction in Arl8B-positive puncta density for Arl8B shRNA-expressing axons to about a third of the density in axons expressing the control shRNA (Fig. 3F).

Arl8B controls the density and location of lysosomes in RGC axons
Arl8B is located on lysosomes (Fig. 1), where it promotes their anterograde transport as an adaptor molecule to kinesin motor proteins (Garg et al., 2011; Rosa-Ferreira and Munro, 2011; Tuli et al., 2013; Pu et al., 2015; Farias et al., 2017; Marwaha et al., 2017), in particular by relieving the autoinhibition of kinesins (Niwa et al., 2016).

We therefore investigated whether the observed proximo-distal shift in relative axon branch position in response to altering Arl8B levels can be correlated with a similar shift in lysosome position (Fig. 4). Retinal cultures were transfected with expression vectors for Arl8B-GFP or control protein (GFP), together with small amounts of a LAMP1-RFP expression vector, to specifically label lysosomes. Three days later, the density of LAMP1-positive puncta and their relative position along RGC axons were analyzed (Fig. 4A–G).

Our data show that Arl8B overexpression results in a ~2-fold overall increase in lysosome intensity compared with controls (Fig. 4E). We found a particular increase in LAMP1-RFP...
fluorescence in the distal axonal compartment, as well as a reduction in fluorescence in the proximal compartment (Fig. 4G). Overexpression of Arl8B did not affect axon length (Fig. 4F).

In complementary experiments (Fig. 4H–L), we found that Arl8B knock-down reduces the overall LAMP1-GFP fluorescence in RGC axons (Fig. 4J), while axon length was not affected (Fig. 4K). Intriguingly, Arl8B knock-down increases the LAMP1-RFP fluorescence in the proximal part of axons (Fig. 4L).

In sum, underscoring its function as an adaptor molecule linking lysosomes and kinesins, we show here that overexpression of Arl8B results in an overall increase in lysosome density and their accumulation in the distal part of RGC axons, while Arl8B knock-down leads to a decrease in the density of lysosomes and their accumulation in the proximal parts of axons.

Thus, our data unravel a striking correlation between lysosome positioning and density, on the one hand, and axon branch positioning and density, on the other hand.

**Arl8B controls autophagosome density and location**

Towards unravelling the mechanisms by which Arl8B-mediated positioning of lysosomes might play a role in positioning interstitial axon branches, we analyzed whether changing the expression level of Arl8B also affects the density or distribution of other components of the autophagy pathway (Fig. 5). For this, we expressed a mCherry-tagged version of LC3, a central protein for autophagosome biogenesis, in retinal neurons enabling us to visualize the formation of autophagosomes, a process that is considered a hallmark of induction of autophagy (Klionsky et al., 2012). The mCherry-LC3 protein normally shows a predominantly cytoplasmic and diffuse staining pattern (LC3-I); however, it is (cleaved and) lipidated during autophagy induction and incorporated into forming autophagosomes (LC3-II) resulting in a punctate (fluorescence) pattern (Klionsky et al., 2012). Thus, the density of puncta of LC3-II can be taken as a read-out for the level of autophagy (Klionsky et al., 2012).

We found that expression of only mCherry-LC3 in RGC axons, that is, at baseline levels of Arl8B, leads to a low density of fluorescent puncta (Fig. 5A,C), indicating that RGC axons normally contain only a small number of (steady-state) autophagosomes. However, after overexpression of Arl8B, we observed a dramatic ~3-fold increase in the number of fluorescent mCherry-LC3 puncta (Fig. 5B,C). This increase was particularly manifest in the distal part of RGC axons, while the density of puncta in the proximal part was reduced compared with controls (Fig. 5E). These findings are in good agreement with other reports which showed that overexpression of Arl8B in fibroblast cells leads to an increase in LC3-II levels, i.e., an increase in autophagy (Korolchuk et al., 2011).

Thus, these data show that Arl8B expression levels affect not only the spatial distribution of lysosomes but also the spatial distribution of autophagosomes, such that areas of higher autophagosome density are areas of higher axon branch density. This ultimately suggests that local activation of autophagy is involved in branch formation.

**Baseline levels of autophagy are necessary for retinal axon branching**

Based on this intriguing correlation between Arl8B-induced axon branching and autophagosome/lysosome distribution, we next asked whether abolishing or increasing autophagy directly would also affect retinal axon branch density.

To study the effects of abolishing autophagy, we knocked down Atg7, a protein whose function is essential for the initiation of autophagosome formation, and which is a validated target for disrupting autophagy (Komatsu et al., 2006, 2007). Using an miR-like shRNA approach (Shan et al., 2009), we identified shRNAs which efficiently knocked down expression of the Atg7 protein (Fig. 6A,B). To verify that this Atg7 shRNA indeed affects autophagy, we cotransfected Atg7 shRNA and mCherry-LC3 and found a significant reduction in LC3-II puncta density compared with control shRNA transfected neurons (Fig. 6C). These results are comparable to an analysis of brain tissue from...
mice with a full knock-out of Atg7, which showed a lack of formation of LC3-II (Komatsu et al., 2006).

In this series of experiments, we found that knock-down of Atg7 led to a reduction in overall axon branch density to 52.0 ± 6.52% (mean ± SEM; Fig. 6D) compared with cultures transfected with control shRNAs (100 ± 10.54%; mean ± SEM). This indicates that autophagy is affecting retinal axon branching.

Furthermore, we did not find statistically significant shifts in axon branch location between neurons transfected with Atg7 shRNA compared with control shRNA. In case of the proximal and distal axonal thirds, in proximal axon branch location between neurons transfected with Atg7 shRNA compared with control shRNA (100 ± 52.0% of 30.6 ± 5.62% and 75 ± 5.62%). Axon length was not affected (Fig. 6E), and that bafilomycin might have an effect also on other biological processes.

In parallel experiments, we analyzed whether bafilomycin at the concentration tested here leads to an abolishment/reduction in autophagy in retinal cultures (Gomez-Sintes et al., 2017). For this, we analyzed again the expression level of LC3-II (Klionsky et al., 2012); however, this time using Western blot analyses (Fig. 7C,D). We found a good correlation between the concentrations of bafilomycin-A1 in the retinal cultures and the level of LC3-II, meaning that retinal cultures at these developmental stages in fact show basal levels of autophagy (Boya et al., 2016) and that bafilomycin-A1 abolishes autophagic flow, i.e., a prevention of acidification of lysosomes prevents their fusion with autophagosomes and thus abolishes LC3-II degradation.

**Figure 6.** Autophagy controls axon branching. Increasing or decreasing autophagy results in an increase or decrease in RGC axon branch density, respectively. A, B, Characterization of shRNAs for ATG7. A, Plasmids for expression of Atg7Flag, and either Atg7 shRNA or a control (scrambled) shRNA plasmids were cotransfected into CHO cells. Three days later, the lysates were investigated in a Western blot analysis using an anti-FLAG antibody and for a loading control, an anti-α-tubulin antibody. B, Corresponding quantification from three independent experiments. Error bars represent SEM. A vs B: n = 7.4%; reduced to 91.9 ± 0.6% using Student’s t test; ***p < 0.001 and *p < 0.05; n.s., not significant.

**C–E.** Single-cell cultures from E8 dissociated chick retina were transfected with shRNAs against Atg7 to block autophagy, or with a control shRNA. C, Analysis of retinal cultures after transfection of Atg7 shRNA and a plasmid for GFP-LC3 shows a downregulation of LC3-II puncta density compared with transfection with control shRNAs. D, Reduction in axon branch density in axons transfected with Atg7 shRNA compared with controls. Axon length was not affected (E). C–E, Error bars denote SEM. Statistical analyses were done using Student’s t test; ***p < 0.001 and *p < 0.05; n.s., not significant.

**Induction of autophagy increases RGC axon branching**

In the complementary approach, we investigated whether increasing autophagy leads to an increase in axon branch density (Fig. 7E–G). We used an approach that is based on findings showing that an activated mTORC1 pathway blocks autophagy. Treatment of retinal cultures with rapamycin, which inactivates the mTOR pathway, should therefore relieve this block, leading in turn to an increase in autophagy. To verify this, we transected retinal cultures with LC3-mCherry, and analyzed the density of LC3-II-positive puncta in RGC axons after treatment with rapamycin (Fig. 7E; see Materials and Methods). We found that overnight treatment with 20 nM rapamycin significantly increased LC3-II puncta density in RGC axons (Fig. 7E), indicating that rapamycin indeed increases autophagy in these cells.

Using the same experimental paradigm, we then analyzed axon branch density. Our results show that treatment with 20 nM rapamycin overnight led to a statistically significant increase in axon branch density, compared with controls treated with DMSO only (Fig. 7F). Axon length was not affected (Fig. 7G).

Thus, our data are consistent with the idea that upregulation and downregulation of autophagy lead to an upregulation and downregulation of RGC axon branch density, respectively.

**Arl8B controls axon branching through autophagy**

To further scrutinize the link between Arl8B, axon branching and autophagy, we overexpressed Arl8B, but then asked whether the associated increase in axon branching (Fig. 2) could be eliminated by abolishing autophagy via knock-down
of Atg7, which decreases axon branches (Fig. 6). If this decrease were not observed, it would suggest that Arl8B controls axon branching using a pathway independent of autophagy.

However, our data show in fact that the Arl8B-induced increase in branch density can be abolished by a knockdown of Atg7 (Fig. 7H) and is therefore in line with our principal hypothesis that Arl8B controls axon branching via lysosome/autophagosome positioning. Additionally, overexpression of Arl8B in the presence of rapamycin did not lead to a statistically significant increase in axon branching compared with overexpression of Arl8B alone (Fig. 7H).

Restricted colocalization of pre-SV clusters with Arl8B-positive vesicles, autophagosomes, and lysosomes

In view of the synaptotropic hypothesis which proposes a link between axon branching and presynapse formation (see Introduction), we then analyzed (1) whether pre-SV clusters colocalize with Arl8B, and (2) whether alterations in Arl8B expression level also shift the axonal position of pre-synapses, similar to the effects shown for lysosomes (Fig. 4) and autophagosomes (Fig. 5).

To label pre-SV s, we used an expression plasmid for synaptophysin tagged with GFP, syn-GFP. A confocal analysis of RGC axons 2 d after transfection of small amounts of respective expression plasmids showed a small colocalization between Arl8B-tdT puncta and syn-GFP puncta (Fig. 8A–C). A quantification by intensity correlation analysis using the ImageJ plugin ‘coloc’ (see Materials and Methods) demonstrates that this colocalization shows a statistically significant correlation (Pearson’s correlation, \( r = 0.45 \); see Materials and Methods).

Although we observed only a limited synaptophysin/Arl8B colocalization, we investigated whether pre-SV distribution would change in response to an increase or decrease in the expression of Arl8B, aimed to uncover a (possible) link between positioning of pre-SV clusters and axon branches. We found that a downregulation of Arl8B using shRNA did not alter the relative SV2-puncta distribution along RGC axons compared with controls (Fig. 8D,E); however, a small decrease in the density of synaptophysin-positive puncta in the proximal third of axons was observed after Arl8B overexpression (Fig. 8D), suggesting rather an anti-correlation between positioning of pre-SV clusters and axon branching.

We also investigated a (possible) colocalization of pre-SV clusters with lysosomes and autophagosomes (Fig. 9). To monitor this, we transfected LC3-GFP into retinal cultures (Fig. 9A–F), which were then treated with bafilomycin-A1 to increase the likelihood of detecting LC3-positive puncta.
Similar data were obtained for a colocalization between SV2 and lysosomes with a Pearson’s correlation of \( r = 0.12 \) (Fig. 9G–L).

In sum, these studies show a rather limited colocalization between pre-SV clusters on the one hand, and Arl8B, autophagosomes and lysosomes on the other hand. In fact, changing expression levels of Arl8B did not, or only little, affect pre-SV cluster positioning, which is in clear contrast with the obvious and consistent changes in autophagosome and lysosome positioning following Arl8B mis-expression. These results therefore suggest that Arl8B mediates its effect on axon branch density/positioning mainly through controlling (locally) autophagy-related processes but not through shifting pre-SV clusters.

**Autophagy is necessary for proper formation of the retino-collicular projection**

Next, we investigated whether interference with autophagy *in vivo* affects axon branching (Fig. 10), for which we used the mouse retino-collicular projection as a model, where RGC axons project from the eye to their main principle midbrain target, the superior colliculus (SC), in a topographic manner (for review, see Suetterlin et al., 2012; Cang and Feldheim, 2013). Based on our *in vitro* data (Fig. 6), the expectation was that blocking autophagy *in vivo* would lead to a reduction in RGC axon branching in the SC and thus a decrease in densities of the detected TZs.

For this approach, we used a mouse line in which the *atg7* gene, whose gene product is essential for autophagy and was targeted in our *in vitro* experiments (Fig. 6), can be conditionally inactivated using the cre/loxP system (Komatsu et al., 2006, 2007). These mice were crossed with a mouse line in which the Cre recombinase is expressed under control of the \( \alpha \)-enhancer of the pax6 promoter whose activity is restricted to the nasal and temporal parts of the retina (\( \alpha \)-pax6:cre; Marquardt et al., 2001). In homozygous *atg7\(^{fl/fl}\); cre\(^{+}\) offspring, autophagy is abolished only in these regions, leaving an unaffected area in the middle of the retina (Burbridge et al., 2014).

To confirm the expression of Cre in \( \alpha \)-pax6:cre mice, we crossed these mice into a floxed-stop tdTomato reporter line (Aii4; https://www.jax.org/strain/007914) and verified the expression of tdTomato restricted to the temporal and nasal retina (Fig. 10A) and the projection pattern of (tdTomato-positive) temporal and nasal RGC axons to the rostral and caudal SC, respectively (Fig. 10B; Burbridge et al., 2014).

We then analyzed the offspring from crosses between heterozygous *atg7\(^{fl/+}\); cre\(^{+}\) mice (*atg7\(^{fl/+}\); cre\(^{+}\) x *atg7\(^{fl/+}\); cre\(^{+}\)). First, we investigated retina development in the offspring from these crosses (Fig. 10C–F), and found an apparently undisturbed, normal development of all retinal layers including the RGC layer in *atg7\(^{fl/+}\); cre\(^{+}\) mice when compared with *atg7\(^{fl/+}\); cre\(^{+}\) mice. A quantification of the fluorescence intensity of the DAPI stained RGC layer along the naso-temporal axis showed no apparent differences between nasal/temporal versus central regions, neither within or between *atg7\(^{fl/+}\); cre\(^{+}\) and *atg7\(^{fl/+}\); cre\(^{+}\) genotypes (Fig. 10E–F). This finding shows that the RGC layer contains similar numbers of cells suggesting that inactivation of autophagy does not affect normal development of RGCs at least up to P9.

Next, we investigated the projection pattern of nasal axons in the offspring from these crosses (Fig. 10G–L). For this, we injected a constant small amount of the lipophilic axon tracer DiI into the peripheral part of the nasal retina at postnatal day (P8). The corresponding TZs in the SC were analyzed 1 d later at a developmental stage at which the retina-collicular projection is
considered to be mature (Sutterlin and Drescher, 2014). Typical images from whole mounts (Fig. 10G, H) and parasagittal sections through the SC are shown (Fig. 10I, J). Using whole mount images, we then quantified the density of DiI-labeled TZs, defined as the sum of pixel intensities of the TZ divided by the area of the TZ (for details, see Materials and Methods) assuming that pixel intensity directly correlates with the amount of DiI-labeled axonal branches (Fig. 10K, L).

Our analysis showed that the TZ density in atg7fl/fl; cre+ mice (n = 17) was significantly reduced to 53.7 ± 7.1% (mean ± SEM) when compared with controls [atg7fl/fl; cre−; n = 11; 100 ± 17.6% (mean ± SEM); p < 0.05; Fig. 10G–J]. The reduction in the density of the TZs suggests that inactivation of autophagy in the nasal retina results in a diminished connectivity of RGC axons in the SC, which correlates well with our results obtained in in vitro approaches (Fig. 6). These two sets of data support the view that the reduction in TZ branch density in atg7fl/fl; cre+ mice compared with atg7fl/fl; cre− mice is because of a disrupted connectivity pattern of RGC axons but not a defective retina development.

Discussion

Interstitial axon branching is a key step during the formation of neural circuits. Here, we have investigated the function of the GTPase Arl8B in this process, an adaptor molecule linking lysosomes to kinesin motor proteins. We show that up and down regulation of Arl8B leads to changes in the density and position of lysosomes and autophagosomes, and this together leads to changes in axon branch density and position. Direct abolition of autophagy reduces branch density, while increasing autophagy leads to an increase in branch density. In vivo, disruption of autophagy in the retina leads to a disturbance of the retino-collicular projection. Our data suggest that Arl8B controls the location of axon branches by controlling positioning of lysosomes and autophagosomes, that is by controlling local autophagy.
Correlation between Arl8B-mediated location of axon branches and lysosome positioning

Our principle finding that Arl8B, a GTPase specifically bound to lysosomes, is prominently involved in axon branch formation provides an entirely new avenue to understand the intracellular signaling controlling this process suggesting that in fact lysosomes are involved in this process.

Lysosomes exert multiple functions, and are not only involved in autophagy, but contribute to other processes including cell migration, formation of focal adhesions and membrane repair (for review, see Pu et al., 2016). Therefore, we characterized which of these canonical lysosome function/s are involved in controlling axon branching. For this, we demonstrate that also a direct interference with autophagy affects axon branching density, that is a decrease in autophagy reduces the density of RGC axon branches (Fig. 6), while an upregulation of autophagy increases branch density (Fig. 7). Furthermore, the Arl8B-mediated increase in branching could be abolished by knock-down of autophagy (Fig. 7H). This makes it likely that Arl8B is involved in axon branching by controlling autophagy-related lysosome functions.

Interestingly, autophagy is also required for dendritic branching (Clark et al., 2018); however, the mechanisms controlling dendritic branching must be different from those controlling axonal branching since Arl8B is not transported into dendrites (Farias et al., 2017).

An additional argument supporting our hypothesis is our finding that branching and autophagy are locally correlated, as visualized by the distribution of autophagosomes, which serve as a hallmark of autophagy. We found that upregulating Arl8B expression leads to an increase in lysosome and autophagosome density in the distal part of axons (Figs. 4, 5), and therefore in the same region as we detect an increase in axon branching. Similarly, downregulation of Arl8B leads to an increased density of lysosomes in the proximal part of axons (Fig. 4) in parallel to an increase in axon branches in the same region (Fig. 2).

Arl8B controls branching via autophagosome/lysosome interactions

How might an increased Arl8B expression lead to a higher density of autophagosomes in the distal axon? Normally, lysosomes preferentially form at the cell
body and are from here transported via Arl8B/kinesins/MTs into the axon, while autophagosomes form preferentially in the distal parts of the axon (Hollenbeck, 1993; Lee et al., 2011; Maday et al., 2012; Maday and Holzbaur, 2014; Cheng et al., 2015). Autophagosomes are retrogradely transported toward the cell body only after their fusion with lysosomes (autophagic flow; Maday et al., 2012). It is the fusion between lysosomes and autophagosomes which leads to an altered (activity) balance of motor proteins on these maturing autolysosomes which results in their retrograde transport back to the cell body.

After overexpression of Arl8B and in view of the function of Arl8B in relieving the kinesin autoinhibition, lysosomes appear to be linked to a much higher kinesin activity, i.e., a higher anterograde transport activity. Their fusion with autophagosomes would shift now the balance toward an anterograde transport, i.e., prevent a retrograde transport, and might therefore be the reason for the higher density of autophagosomes in the distal part of axons (Fig. 5), which is linked to a higher branch density.

Possible mechanism of axon branch induction by local autophagy

Based on the general concept that RGC axons are normally refractory to branching, we hypothesize that branching will occur only in regions where “suppressors” of axon branching have been removed by local (selective) autophagy (Fig. 11 A–C). Thus, branching proceeds at locations devoid of suppressors (Fig. 11 D). These locations will be determined by a set of factors produced by tectal cells, such as branch-promoting BDNF/TrkB and branch-suppressing Epha/ephrinA activities (Marler et al., 2008; Fig. 11).

Since debundling of MTs is a defining and early feature of interstitial branching (Kalil and Dent, 2014; Ketschek et al., 2015), prime intracellular targets of these combined TrkB/EphA signaling cascades could be molecules which normally suppress the debundling of MTs, such as MAP1B. In support of this view it has been shown that removal of MAP1B results in an increase of axon branching (Ketschek et al., 2015; Barnat et al., 2016). Moreover, the phosphorylated form of MAP1B preferentially interacts with autophagosomes (Wang et al., 2006; Yue, 2007), leading to a model in which the activation of upstream signaling cascades leads to the modification of the suppressor MAP1B. This now becomes now a target of autophagy and is removed, which allows axon branches to form and stabilize (Fig. 11).

Colocalization between Arl8B and pre-SV clusters

We found a small, but significant, colocalization between Arl8B-positive puncta (i.e., lysosomes and autophagosomes) and pre-SV clusters. However, we did not observe a change in the distribution of these presynapse clusters after overexpression or downregulation of Arl8B (Fig. 8) in a manner suggesting a correlation with axon branching. This lack of correlation therefore does not support a model in which presynapse formation and autophagy are entangled to control early steps of axon branching.

On the contrary, in a restricted number of neuronal populations in C. elegans, axonal autophagy and synapse development are functionally linked (Stavoe et al., 2016). Here, mutants in genes in the autophagy pathway lead to defects in synapse formation. However, this correlation was not found for most other neuronal classes in C. elegans (Stavoe et al., 2016) and seems therefore not to be a general principle of synapse formation.

Nevertheless, we cannot exclude that branch formation of RGC axons occurs from a sub-population of pre-SV clusters; however, these would be difficult to detect under our experimental conditions.

Basal levels of autophagy are necessary for retino-collicular map formation

Little is known so far about the role of autophagy-related genes in establishing neural connectivity during development in vivo. However, the key autophagy kinases ULK1 and ULK2 do play a role in the development of the forebrain since respective knock-out mutants of these genes show defects in axonal pathfinding and de-fasciculation of major axonal tracts including the corpus callosum and the cortico-thalamic tract (Wang et al., 2018). Defects in formation of the major forebrain commissures were also observed for mutations in alfy (WDFY3), a scaffolding protein which facilitates the sorting of ubiquitinated cargo into autophagosomes (Dragich et al., 2016; Napoli et al., 2018).

We show here now, in good agreement with our in vitro data, that a retina-specific knock-out of autophagy leads to a disturbance of the formation of the retino-collicular projection. Nasal RGC axons from atg7fl/fl; cre- mice showed a significant reduction in the density of their TZs in the SC, compared with atg7fl/fl; cre+ controls (Fig. 11). The restricted expression of Cre in the retina but not in the SC in pax6-acre mice (Fig. 11; Marquardt et al., 2001) allows the conclusion that these connectivity defects are specifically because of a block of autophagy in RGC axons and not because of alterations in the SC.

Outlook

Our in vitro and in vivo data presented here suggest that Arl8B controls the density and positioning of axon branches by regulating the transport of vesicles mediating locally autophagy. Future work will focus on the identification of factors with in turn...
control the activity of Arl8B (Donaldson and Jackson, 2011; Niwa et al., 2017), the corresponding upstream signaling pathways (Marler et al., 2008), and the molecular mechanisms by which autophagy controls neural circuit formation in vivo.

References


Hofmann et al., 2008

Hofmann et al., 2010


