Published in final edited form as:

Dev Neurobiol. 2014 March ; 74(3): 303–318. doi:10.1002/dneu.22110.

RNA binding protein Vg1RBP regulates terminal arbor formation but not long-range axon navigation in the developing visual system

Adrianna Kalous¹, James I. Stake¹, Joel K. Yisraeli², and Christine E. Holt^{1,§}

¹Department of Physiology, Development and Neuroscience, University of Cambridge, CB2 3DY, United Kingdom.

²Faculty of Medicine, Hebrew University, Jerusalem, POB 12272, Israel.

Abstract

Local synthesis of β -actin is required for attractive turning responses to guidance cues of growth cones *in vitro* but its functional role in axon guidance *in vivo* is poorly understood. The transport and translation of β -actin mRNA is regulated by the RNA binding protein, Vg1RBP (ZBP1). To examine whether Vg1RBP plays a role in axon navigation *in vivo*, we disrupted Vg1RBP function in embryonic *Xenopus laevis* retinal ganglion cells (RGCs) by expressing a dominant-negative Vg1RBP and by antisense morpholino knockdown. We found that attractive turning to a netrin-1 gradient *in vitro* was abolished in Vg1RBP-deficient axons but, surprisingly, the long-range navigation from the retina to the optic tectum was unaffected. Within the tectum, however, the branching and complexity of axon terminals were significantly reduced. High-resolution time-lapse imaging of axon terminals *in vivo* revealed that Vg1RBP-GFP-positive granules accumulate locally in the axon shaft immediately preceding the emergence a filopodial-like protrusion. Comparative analysis of branch dynamics showed that Vg1RBP-deficient axons extend far fewer filopodial-like protrusions than control axons and indicate that Vg1RBP promotes filopodial formation, an essential step in branch initiation. Our findings show that Vg1RBP is required for terminal arborization but not long-range axon navigation and suggest that Vg1RBP-regulated mRNA translation promotes synaptic complexity.

Keywords

RNA binding protein; axon guidance; axon branching

INTRODUCTION

Axons and growth cones in the developing nervous system integrate signals from multiple guidance cues to grow appropriately towards, and innervate, their synaptic targets. Studies in

[§]Corresponding author. ceh33@cam.ac.uk. Phone: +44 1223 766229 .
Adrianna Kalous. adrianna.kalous@gmail.com. Phone: +61 02 9904 1077
James Stake. stake.james@gmail.com
Joel K Yisraeli. Joel.Yisraeli@huji.ac.il.

The authors declare no competing financial interests.

cultured axons and growth cones show that guidance cues induce spatially restricted activation of signalling components, to drive localised rearrangements of the cytoskeleton, focal adhesion dynamics, and membrane remodelling (Tojima et al., 2011). Several guidance cues have been shown to require local protein synthesis in the growth cone to elicit chemotropic responses *in vitro* (Campbell and Holt, 2001; Wu et al., 2005; Piper et al., 2006). Attractive gradients of netrin-1 or BDNF rapidly induce accumulation and local translation of β -actin mRNA in embryonic *Xenopus* growth cones on the side apposing the gradient source (Leung et al., 2006; Yao et al., 2006). Similarly, NGF-coated beads trigger localisation and translation of β -actin mRNA and *de novo* formation of branches in rat cultured axons (Willis et al., 2007).

Embryonic axons and growth cones contain a large and diverse pool of mRNAs (Zivraj et al., 2010; Gumy et al., 2011). *In vitro* studies are beginning to reveal how axonal mRNAs are regulated by *trans*-acting factors, but the functional role of axonal mRNA regulation *in vivo* is largely unexplored. β -actin mRNA contains a 54n 'zipcode' element in the 3'UTR that is bound by zipcode binding protein-1 (ZBP1; Kislauskis et al., 1994; Ross et al., 1997), or in *Xenopus* the homologue Vg1RBP (Leung et al., 2006). Zipcode-mediated control of β -actin mRNA localisation and translation by ZBP1/Vg1RBP is one of the best characterised models of subcellular mRNA regulation. ZBP1 deficiency, or expression of non-phosphorylatable ZBP1, which is unable to release bound transcripts for translation (Huttelmaier et al., 2005), prevents attractive growth cone turning responses *in vitro* (Welshhans and Bassell, 2012). Morpholinos that block β -actin mRNA translation or accessibility of the β -actin 3'UTR zipcode, also prevent attractive growth cone turning (Leung et al., 2006; Yao et al., 2006). These findings suggest that the interaction between β -actin mRNA and ZBP1 or Vg1RBP may be critical for axon guidance *in vivo*.

Knockout of ZBP1 in mice is lethal, which has hindered examination of the functional relevance of ZBP1 in axon guidance *in vivo*. Similarly, mice with CNS-specific knock-out of β -actin rarely survive postnatally, but histological analysis of brain architecture in survivors has revealed surprisingly few abnormalities, which are highly restricted to specific brain structures (Cheever et al., 2012). In this study, we test the requirement for Vg1RBP in axon guidance in the embryonic *Xenopus laevis* visual system. We show that Vg1RBP is required for attractive turning responses of RGC growth cones *in vitro*, but is not essential for the long-range navigation of RGC axons from the eye to their synaptic targets in the tectum. We demonstrate that Vg1RBP is involved in terminal branching of RGC axons in the tectum, where it localises to regions of the axon shaft from which filopodia arise and promotes axonal filopodial emergence.

MATERIALS & METHODS

Embryos

X. laevis embryos obtained by *in vitro* fertilization were raised in 0.1X Modified Barth's Saline at 14-18°C. Staging was according to the tables of Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Constructs and Morpholinos

The Vg1RBP pCS2+ constructs used in this study have been described previously (Oberman et al., 2007). The utrophin-mCherry construct was purchased from addgene (plasmid 26740). Membrane-targeted GFP (gapGFP) or mCherry (GAP-mCherry) cloned into pCS2+ vector was used as a negative control or to visualise axons, respectively. Antisense morpholino oligonucleotides conjugated to fluorescein were purchased from GeneTools. *Xenopus* Vg1RBP AMO, 5' AAAGAAGACGAGCCCAA- AAACCCG 3'; control MO, 5' CCTCTTACCTCAGTTACAATTTATA 3'.

Immunohistochemistry

Sections were washed in 1X phosphate buffered saline (PBS), then blocked and permeabilised for 1 hour in 10% heat-inactivated goat serum and 0.1% triton-X100 in PBS. Sections were incubated overnight in rabbit anti-Vg1RBP (1:500; gift from N. Standart, University of Cambridge) or mouse anti-acetylated tubulin (1:600, Sigma) antibodies, followed by washes and 1 hour incubation in goat anti-rabbit Alexa488 or donkey anti-mouse Alexa594 secondary antibodies (both 1:500, Jackson Immunoresearch Laboratories). After further washes, sections were counterstained with DAPI then coverslipped in fluorosave (Calbiochem).

Cultured neurons were fixed for 30 minutes in 2 % formaldehyde and 7.5 % sucrose in PBS, washed in PBS, then permeabilised for 5 minutes in 0.1 % triton X-100. After washes, cultures were blocked for 1 hour in 5 % heat-inactivated goat serum, then incubated for 4 hours in rabbit anti-Vg1RBP (1:200; as before), followed by washes and 1 hour incubation in goat anti-rabbit Alexa-488 (1:500, as before). Washed coverslips were mounted in fluorosave.

Western Blot

Eyes were dissected from stage 33/34 and 45 embryos, and homogenised in a protease inhibitor cocktail diluted in RIPA buffer (Sigma). Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane (BioRad). The membrane was blocked for 30 minutes in 5% skim milk powder in TBST (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.05 % Tween) before incubation in rabbit anti-Vg1RBP (1:5000; as before), and mouse anti- γ -tubulin (1:5000; Abcam) antibodies, followed by horseradish peroxidase-conjugated anti-rabbit (1:5000, Zymed) and anti-mouse (1:5000, Abcam) secondary antibodies. Bands were detected using an ECL Plus detection kit (Amersham) and X-ray developer.

Electroporation

Eyes were electroporated as previously described (Falk et al., 2007). Briefly, the retinal primordia of stage 26-28 embryos were injected with 1 μ g/ml DNA in water, followed by 8 electric pulses of 50 ms duration, delivered at 18 V. For experiments requiring DNA expression restricted to 1-2 axons, primordia were injected at stage 29/30, with the number of electric pulses reduced to 4, delivered at 16 V. Embryos were raised to stages 41-42 and prepared for *in vivo* time-lapse imaging, or fixed in 4 % formaldehyde + 0.1 %

glutaraldehyde overnight at 4°C. Fixed brain samples were mounted lateral side up in glycerol/PBS.

Microinjection

Microinjections were performed using previously described methods. The jelly coats of 2-cell stage embryos were removed using a solution of 2 % cysteine (Sigma) in 0.1X MBS. MOs and RNA were dissolved in water, and microinjected into both dorsal blastomeres at 4-cell stage in a solution of 4 % ficoll in 0.1X MBS.

Dil-labeling

Embryos were raised to stage 41-42 and fixed for 2 hours in 4% formaldehyde at room temperature. DiI dissolved in ethanol was injected into the intra-retinal space of the left eye. Embryos were stored in 0.5% formaldehyde at room temperature for 24 hours until dissection. 'Open-book' brain samples were mounted lateral side up in 1X PBS and imaged immediately.

Optic tract and axon arbor analysis

Mounted brain samples were viewed at 20X and 40X using a Nikon Eclipse upright microscope connected to a Hamamatsu ORCA-ER digital camera. OpenLab software (Improvision) was used to acquire z-stacks through the optic tract or individual axon arbours. Optic tract length was analysed in merged z-stacks by measuring the distance between the chiasm and dorsal border of the axon bundle, normalised to the distance between the chiasm and the isthmus. Branching was analysed by manually tracing each axon through the z-stack to obtain a 2D reconstruction of the arbour, from which branch number and order could be counted. Only protrusions 5 µm or greater in length were counted as branches.

Retinal cultures

Eyes were dissected and plated onto glass coverslips coated with 10 µg/ml poly-L-lysine (Sigma) and 10 µg/ml laminin (Sigma) or 10 µg/ml fibronectin (Calbiochem). Explants were cultured at 20°C in 60 % Leibowitz's L15 medium (Gibco) containing 5 % penicillin/streptomycin/fungizone. Turning assays were performed and analysed as previously described (de la Torre et al., 1997), by placing a pulsing pipette containing netrin-1 (10 µg/ml; R&D Systems) 100 µm away from the growth cone, at an angle of 45° from the direction of growth.

In vivo time-lapse imaging

Stage 41-42 embryos were lightly anaesthetised in 0.3mM MS222 in 1X MBS. The lateral surface of the side of the brain contralateral to the electroporated eye was exposed by carefully removing the overlying eye and skin. Embryos were mounted into oxygenated chambers, with the exposed brain facing the coverslip. Time-lapse imaging of filopodia formation was performed at 60X using a Nikon Eclipse TE2000-U inverted microscope connected to a Hamamatsu ORCA-ER digital camera. Z-stacks of individual axon arbours were acquired at each time point using OpenLab. Z-stack images were taken 1-1.5 µm apart.

Time-lapse imaging of Vg1RBP-eGFP was performed using a 100X water immersion objective with an Olympus 1X81 inverted spinning disk microscope and UltraView VoX confocal imaging system (Perkin Elmer).

RESULTS

Expression of Vg1RBP in *Xenopus* retinal ganglion cells

Previous *in situ* hybridization studies have shown that Vg1RBP mRNA is expressed in the eye, brain, neural tube, and otic vesicle of stage 26 and 30/31 *X. laevis* embryos (Zhang et al., 1999). We performed immunohistochemistry using an antibody against *Xenopus* Vg1RBP to detect the expression pattern in the eye at an early developmental stage when RGC axons are undergoing long-range navigation of optic tract (stage 33/34), and at a later stage when axons are arborizing within the tectum (stage 45). Vg1RBP is expressed ubiquitously in retinal cell bodies at both stages (Figure 1A-F), but levels are elevated at stage 45 compared with stage 33/34, as shown by Western blot analysis of eye lysates using a Vg1RBP antibody (Figure 1G). The plexiform layers of the retina displayed a diffuse Vg1RBP immunoreaction signal (Figure 1D'-F'). To confirm the presence of Vg1RBP in axons and growth cones, we immunostained cultured RGCs with the Vg1RBP antibody, which revealed a dense granular expression pattern in axons and growth cones (Figure 1H-I).

Dominant-negative Vg1RBP does not affect long-range axon navigation *in vivo*

To test whether Vg1RBP is required for long-range navigation of RGC axons from the eye to their target in the brain, we expressed an eGFP-tagged dominant-negative mutant of Vg1RBP (Figure 2A) lacking the fourth KH domain which mediates RNA binding (Vg1RBP KH4-eGFP, hereafter KH4-eGFP; (Git and Standart, 2002; Oberman et al., 2007)). We targeted cDNA expression to retinal neurons *in vivo* by electroporating the eyes of stage 26 embryos, just prior to axonogenesis of RGCs (Falk et al., 2007). We also electroporated cDNAs encoding full-length eGFP-tagged Vg1RBP (Vg1RBP-eGFP) or gapGFP as a control. Each construct was co-electroporated with GAP-mCherry to aid visualization of axons *in vivo*, as the KH4-eGFP and Vg1RBP-eGFP signals were of insufficient intensity in the axonal compartment to be used for direct visualization. By stages 41-42, most RGC axons have navigated along a stereotypic pathway to form the optic tract and have terminated in the contralateral optic tectum. We assessed potential pathfinding errors of RGC axons in wholemount brain preparations, which enables visualisation of the entire contralateral optic projection (the optic tract and tectum). RGC axons expressing

KH4-eGFP or Vg1RBP-eGFP followed a normal trajectory along the optic tract and were of normal length and appearance (Figure 2B, C). Occasionally, KH4-eGFP-expressing axons were seen straying from the optic tract, but similar straying of Vg1RBP-eGFP and gapGFP-expressing axons occurred at the same frequency (data not shown). Within the tectum, however, we observed that embryos electroporated with KH4-eGFP were more likely to have mis-targeted axons overshooting beyond the boundary of terminated RGC axons in the tectum (arrow, Figure 2B). Compared with gapGFP and Vg1RBP-eGFP expression, KH4-eGFP significantly increased the proportion of embryos with overshooting axons (Figure 2D), but did not affect the number of overshooting axons per

embryo (typically 1-2; Figure 2E). The overshoot length in KH4-eGFP-electroporated embryos ranged from 13.5-169.0 μm , similar to that in embryos electroporated with gapGFP (12.5-113.7 μm) or Vg1RBP-eGFP (13.2-122.6 μm). There was no significant difference in average overshoot lengths (Figure 2F). Since wholemount brain preparations only allow the distal part of the pathway (chiasm to tectum) to be analysed, the possibility that errors occurred in the proximal pathway (eye to chiasm) could not be excluded. To address this, we captured images of serial transverse cryosections at the level of the eyes and diencephalon/midbrain, and arranged image montages to reconstruct the entire length of the optic pathway. Axons expressing Vg1RBP-eGFP (not shown) and KH4-eGFP exhibited normal guidance out of the eye and along the optic pathway (Figure 2G) and no pathfinding defects were observed.

Our observations suggest that Vg1RBP is not involved in axon outgrowth or long-range axon navigation. However, since we used the co-transfected GAP-mCherry reporter signal to visualise the KH4-expressing axons, it is possible that some of these axons had low/no expression of KH4-eGFP, thus giving rise to a false negative result. To exclude this possibility, we analysed the expression of the co-electroporated constructs at single-cell resolution in the retinal sections. The co-expression efficiency of mCherry with KH4-eGFP, Vg1RBP-eGFP or gapGFP was close to 100 % in cells in the retina (Figure 3A-E). The expression levels of KH4-eGFP and Vg1RBP-eGFP were highly variable between neurons, as judged by the fluorescent GFP signal intensity, but within each neuron generally matched the level of mCherry expression: i.e. when the mCherry signal was high, so was the eGFP signal. Imaging of mCherry-labelled RGC axons and growth cones in the tectum at high magnification (100X objective) using a spinning disk microscope confirmed the presence of Vg1RBP-eGFP granules in the distal processes of RGCs *in vivo* (Figure 3F-H). The KH4-eGFP signal was more diffuse and generally lacked distinct puncta (Figure 3I-K). This likely reflects a disruption of assembly into ribonucleoprotein (RNP) complexes, as recognition of mRNA by RBPs is a critical first step in the formation of transport RNPs (Kress et al., 2004). These results confirm that false identification of axons as KH4-eGFP- and Vg1RBP-eGFP-expressing was minimal in our experiments, and does not account for the finding that long-range (eye to tectum) axon navigation occurs normally despite disrupted Vg1RBP function.

To further confirm this finding, we validated the dominant-negative activity of KH4-eGFP in our system by testing its ability to block translation-dependent responses of cultured *X. laevis* RGC growth cones to guidance cues. Previous work has shown that turning of cultured RGC growth cones towards a diffusible gradient of netrin-1 is mediated by local translation of β -actin mRNA and causes the polarised accumulation of Vg1RBP and β -actin mRNA in filopodia on the near-side of the growth cone (Leung et al., 2006). We microinjected the dorsal blastomeres of 4-cell stage embryos with KH4-eGFP mRNA (500 pg / blastomere) to achieve uniform expression of the dominant-negative mutant in retinal neurons, then cultured eyes at stage 23-24 and tested the turning responses of RGC growth cones to netrin-1 (10 $\mu\text{g}/\text{ml}$ in the pipette) in a standard turning assay (Figure 4A-D). The majority of control growth cones exhibited biased growth towards a gradient of netrin-1 (mean turning angle $12.87^\circ \pm 5.84^\circ$; Figure 4F, H), whereas a gradient of the vehicle

solution (0.1% BSA in PBS) did not stimulate any bias in the direction of growth (mean turning angle $-5.75^\circ \pm 5.81^\circ$; Figure 4E, H). By contrast, growth cones expressing KH4-eGFP were not attracted to netrin-1 and, in fact, displayed a bias to grow away from the direction of the gradient source (mean turning angle $-18.40^\circ \pm 4.77^\circ$; Figure 4G, H), although this bias was not statistically significant. These results validate the dominant-negative activity of KH4-eGFP, and also confirm that Vg1RBP is required for attractive growth cone turning *in vitro*.

Vg1RBP knockdown does not affect long-range axon navigation *in vivo*

To verify that Vg1RBP is not required for long-range navigation, we used a knockdown approach to disrupt Vg1RBP function in RGCs *in vivo*. We microinjected 4-cell stage embryos with a fluorescein-tagged antisense morpholino oligonucleotide (AMO) to specifically block Vg1RBP translation. A standard control morpholino oligonucleotide (CMO) was used as a control. Microinjection of the AMO (6 ng/blastomere) at a dose used in a previous study resulted in several developmental abnormalities as reported (Yaniv et al., 2003), including reduced pigmentation, curved neural tube, dramatically smaller or absent eyes, and smaller brains (not shown). In subsequent experiments, we used a lower dose (3 ng/blastomere) that resulted in a minimal reduction in eye and brain size, and no other obvious abnormalities (Figure 5A, B). Eye size was also reduced in embryos microinjected with KH4-eGFP mRNA (see previous), indicating that this effect of the AMO is specific. We never observed a reduction in eye size after electroporation with KH4-eGFP, since expression was restricted to later stages of eye development. The AMO achieved a 50% reduction in Vg1RBP levels, confirmed by Western blot analysis of Stage 33/34 eye lysates using a Vg1RBP antibody (Figure 5C). Despite the smaller eye size, the laminar organisation of the retina appeared normal in AMO-injected embryos (Figure 5D, E), although the thickness of the optic nerve head (ONH) was reduced, likely reflecting a reduction in RGC number (Figure 5D', E').

Following injection of morpholinos, embryos were raised to stage 41-42 then fixed, and retinal axons labelled anterogradely by injecting the left eye with DiI (Figure 5F). Similar to KH4-eGFP-expressing axons, Vg1RBP-depleted axons formed an optic tract of normal length and appearance (Figure 5G). The axon bundle often appeared less dense in AMO-injected embryos, most likely a reflection of the smaller eye size. Occasional axons strayed from the tract in both AMO- and CMO-injected embryos, but quantification did not detect an effect of the AMO on the frequency of these straying axons (data not shown). Axons forming the retinofugal projection to the basal optic nucleus were observed in many samples (arrowheads, Figure 5G), and these were not included in our analyses. Within the tectum, stray axons overshooting past the boundary of terminated retinal axons were observed in $27.26 \pm 4.22\%$ of control embryos; AMO injection more than doubled the proportion of embryos with overshooting axons to $58.45 \pm 3.45\%$ ($P = 0.028$, Fisher's exact test). However, AMO injection did not affect the average number of overshooting axons per embryo (CMO, 1.36 ± 0.17 ; AMO, 1.62 ± 0.18 , $P = 0.241$, Mann Whitney test), or the average overshoot length (CMO, $31.72 \pm 6.17 \mu\text{m}$; AMO, $51.87 \pm 7.54 \mu\text{m}$, $P = 0.114$, Mann Whitney test). Thus, Vg1RBP knockdown results in the same phenotype as dnVg1RBP expression, supporting the conclusion that Vg1RBP is not essential for axon

outgrowth or guidance decisions during long-range navigation *in vivo*. However, the increased probability of axon overshooting in embryos with disrupted Vg1RBP function raises the possibility that Vg1RBP may have a role in short-range guidance during target innervation.

Disruption of Vg1RBP reduces retinal axon branching *in vivo*

Both BDNF and netrin-1 are expressed abundantly in the developing optic tectum and each has been shown to be potent modulators of retinal axon terminal branching (Cohen-Cory, 1999; Manitt et al., 2009). Furthermore, both of these cues are known to require local translation of β -actin mRNA to elicit chemotropic responses in cultured retinal growth cones (Leung et al., 2006; Yao et al., 2006). This prompted us to investigate whether Vg1RBP might be required for terminal branching of retinal axons in the tectum. We co-electroporated eyes at low efficiency to co-express the mCherry-reporter and KH4-eGFP in only one or two RGCs to enable visualisation of the entire terminal arbor of individual axons in the tectum at stage 41-42. Quantitative analysis showed that the average number of branches was significantly reduced in axons expressing KH4-eGFP compared with gapGFP or Vg1RBP-eGFP (Figure 6A, B). Furthermore, KH4-eGFP-expressing axons had an increased proportion of primary branches and a corresponding decrease in the proportion of secondary branches, indicating a reduction in branch complexity (Figure 6C). Similarly, AMO-injection reduced the average number of branches per axon and branch complexity, compared with CMO-injection (Figure 6A, D, E).

A reduction in branch number and complexity could potentially be due to impaired axon extension rate leading to delayed arrival of axons in the tectum and delayed onset of branching. To address whether these parameters were affected, we first examined the requirement for Vg1RBP in axon extension *in vitro*. Blastomeres were injected with mRNA encoding KH4-eGFP or gapGFP at 4-cell stage, then eyes were dissected at stage 33/34 and plated on to poly-L-lysine and laminin-coated coverslips. Approximately 20-24 hours after plating, axons that had extending growth cones were selected and imaged over time-lapse using phase contrast optics. To minimize photo-toxicity, the expression of mRNA in imaged axons was confirmed after imaging by checking the fluorescent signal. There was no significant difference in the mean extension rate of axons expressing KH4-eGFP and gapGFP (μm extended/20 min, gapGFP 13.68 ± 1.62 vs. KH4-eGFP 13.86 ± 1.34 , $n = 8$ axons per group, $P = 0.933$, t test). Furthermore, we did not observe any obvious abnormalities in growth cone morphology, and there was no significant difference in the number of growth cone filopodia in KH4-eGFP and gapGFP-expressing axons (gapGFP 8.69 ± 1.07 filopodia vs. KH4-eGFP 7.0 ± 1.08 filopodia, $n = 8$ axons per group, $P = 0.285$, t test). To further confirm that growth cone morphology and axon extension is not dependent on Vg1RBP or local translation, we performed the same analyses in the presence of the protein synthesis inhibitor, anisomycin. Control axons were imaged for 20 minutes to determine the baseline rate of axon extension, before adding anisomycin ($40 \mu\text{M}$) to the culture medium and imaging the same axon for a further 20 minutes. Anisomycin did not affect the mean axon extension rate (μm extended/20 min, pre-anisomycin 17.79 ± 2.61 vs. post-anisomycin 15.16 ± 2.73 , $n = 9$ axons, $P = 0.279$, paired t test), or the number of growth cone filopodia (pre-anisomycin 6.22 ± 0.86 vs. post-anisomycin 5.22 ± 0.85 , $P =$

0.135, paired t test). These observations indicate that growth cone morphology and extension are not dependent on Vg1RBP or local translation.

We next assessed retinal axon arrival in the tectum by measuring the length of the optic tract at stage 37-38, when the first axons reach the tectum. There was no evidence of a developmental delay, with no difference in tract length in AMO-injected or KH4-eGFP-electroporated embryos compared with respective controls (normalised tract length; CMO 0.61 ± 0.02 vs. AMO 0.53 ± 0.02 ; gapGFP 0.45 ± 0.04 vs. KH4-eGFP 0.52 ± 0.03 , Mann Whitney nonparametric tests, $P > 0.05$). These findings indicate that there is no delay in axon arrival at the tectum and support the conclusion that Vg1RBP has a *bona fide* role in terminal axon branching *in vivo*.

Imaging of dynamic movements of Vg1RBP-eGFP granules and F-actin during *de novo* axonal filopodia formation *in vivo*

In cultured RGCs, Vg1RBP transports and regulates the polarised translation of β -actin mRNA that is required for growth cone turning (Leung et al., 2006; Yao et al., 2006). Since our *in vivo* observations indicate that Vg1RBP is required for axon terminal branching, we hypothesised that branch formation similarly involves localisation of β -actin mRNA by Vg1RBP and accumulation of locally synthesised β -actin. To test this hypothesis, we first performed *in vivo* time-lapse imaging of RGC axons expressing utrophin-mCherry to label the dynamics of filamentous actin (F-actin), to assess whether actin accumulation is associated with branch formation. Utrophin-mCherry signal was most abundant in growth cones and branches in RGC axons *in vivo*, and patches of utrophin-mCherry signal were also present along the axon shaft. We observed both stationary patches and patches that moved in anterograde and retrograde directions along the shaft. This expression pattern was similar to that observed using a β -actin-mCherry construct (not shown).

From a total of 7 axons imaged for 5 minutes at 9 second intervals, we observed 17 filopodia-like protrusions emerging from the axon shaft. Eight of these protrusions (47.1 %) were associated with a local accumulation of utrophin-mCherry that coincided with a bulging of the axon shaft preceding emergence (Fig 7A). The time between signal accumulation and protrusion emergence was rapid, ranging 9-36 seconds. The remaining 9 protrusions (52.9 %) emerged from regions of the axon shaft that were rich in utrophin-mCherry prior to imaging. These observations indicate that axonal protrusions arise from regions of axon shaft with an accumulation of F-actin. However, we did not observe a correlation between F-actin accumulation and protrusion lifetime. We were able to track the lifetimes of 12 protrusions, which were within the duration of the imaging time-course. Lifetimes ranged from 18-144 seconds, averaging 83.67 seconds. In 5 out of 12 (41.7 %) protrusions, removal coincided with dissipation of utrophin-mCherry, 4 (33.3 %) protrusions persisted after dissipation of utrophin-mCherry, and 3 (25 %) protrusions were removed despite a persistence of utrophin-mCherry. These observations suggest the emergence of axonal filopodia-like protrusions requires local accumulation of F-actin, but that subsequent removal is not dependent on F-actin dynamics.

We next attempted to image the dynamics of Vg1RBP-eGFP granules in branching RGC axons in the tectum, to examine if Vg1RBP accumulation is required for branch formation.

Axons were imaged for 5 minutes at 9 second intervals. Because many Vg1RBP-eGFP granules were below detection using the low laser power and short exposure required to minimise photo-bleaching and photo-toxicity, we were unable to assess the correlation between branch formation and Vg1RBP accumulation. However, we did observe clear examples of local Vg1RBP-eGFP accumulation preceding the emergence of axonal protrusions. In the example shown in Figure 7B, two distinct filopodia-like protrusions emerge from the axon shaft at $t = 0$ (arrowheads). One of these protrusions extends and persists throughout the duration of imaging. The other protrusion, and another that emerges at $t = 30$, appear transiently. Local accumulation of Vg1RBP-eGFP can be seen just prior to the emergence of each protrusion. Together with our observations of F-actin dynamics, these findings are consistent with hypothesis that branch formation requires localisation of Vg1RBP and accumulation of β -actin.

Dominant-negative Vg1RBP reduces formation of axonal filopodia-like extensions *in vivo*

We next further investigated the role of Vg1RBP in branching by analysing the branching dynamics of KH4-eGFP-expressing RGC axons in the tectum using time-lapse imaging. *Xenopus* RGC axon extension slows upon arrival in the tectum, when lateral extensions emerge from the axon shaft behind the growth cone, referred to as ‘back-branching’ (Harris et al., 1987; Shirkey et al., 2012). During maturation of the terminal arbor, many extensions are added and removed, with only a small proportion stabilising to become part of the final arbor. Reduced branch number in RGC axons with disrupted Vg1RBP function could reflect a reduction in branch addition or an increase in branch removal. To determine the underlying defect, we imaged axons in the tectum for 10 minutes at 30-second intervals and analysed the number of addition and removal events in RGC axons expressing gapGFP and KH4-eGFP (Figure 8A). An addition event was defined as the emergence of a discernable protrusion from the axon shaft, regardless of protrusion length or lifetime, and a removal event was defined as the complete disappearance of a discernable protrusion from the axon shaft. Axons expressing gapGFP displayed similar addition and removal rates (Figure 8B).

KH4-eGFP expression reduced both addition and removal rates by approximately 70% compared with the respective rates in gapGFP axons, but relative levels of addition and removal remained similar (Figure 8B). These findings demonstrate that Vg1RBP promotes the addition, but not subsequent removal, of axonal protrusions *in vivo*.

DISCUSSION

This study is the first to investigate the functional role of Vg1RBP in axon guidance *in vivo*. Our analyses reveal that Vg1RBP promotes terminal axon branching *in vivo*, but is not required for long-range navigation. Long-range navigation of axons to their synaptic targets is mediated by molecular guidance cues expressed along the pathway, which attract or repel growth cones to direct extension. Upon arrival at the target, the program of pathfinding switches to arborization and synapse formation. Many guidance cues have bifunctional roles in pathfinding and target innervation. For example, netrin-1 expressed in the ONH and tectum directs RGC axons out of the eye and promotes branching, respectively (Deiner et al., 1997; Shewan et al., 2002; Manitt et al., 2009; Shirkey et al., 2012). Thus, it is perhaps

surprising that loss of Vg1RBP function in RGC axons does not result in defective guidance out of the eye.

Redundant guidance signals along the optic pathway may compensate for disruption of Vg1RBP function in RGC axons *in vivo*, accounting for the lack of pathfinding defects. In support of this, netrin-1-deficient mice display only partial disruption of axon entry into the optic nerve head, suggesting that additional cues are involved in guiding RGC axons out of the eye (Deiner et al., 1997). Guidance cues important for pathfinding further along the tract, such as Semaphorin 3A and Slits, are known to elicit translation-dependent responses *in vitro* (Campbell and Holt, 2001; Wu et al., 2005; Piper et al., 2006), but it is not known whether Vg1RBP or ZBP1 is required for these responses. Other RBPs, such as Fragile X mental retardation protein, regulate translation-dependent responses to Sema3A (Li et al., 2009), which might account in part for the insensitivity of long-range navigation to a lack of Vg1RBP function. Our results do not exclude the possibility that Vg1RBP has a role in long-range guidance in other pathways, such as commissural axon guidance. Furthermore, *Xenopus* VgRBP is one of three mammalian gene homologs (IMP1/ZBP1, IMP2, IMP3/Vg1RBP), therefore, the results of the present study leave open the possibility of a role for the IMP1/ZBP1 homolog in axon guidance in mammals.

Our findings could also be explained by age- and substrate-dependent responses of growth cones to guidance cues, which may differentially require Vg1RBP. Age-associated or laminin-induced decreases in cAMP levels convert netrin-1-induced attraction to repulsion (Hopker et al., 1999; Shewan et al., 2002). Similarly, BDNF-induced attraction can be converted to repulsion by inhibiting PKA (Song et al., 1997). While both attractive and repulsive responses to netrin-1 and BDNF are sensitive to protein synthesis inhibitors (Campbell and Holt, 2001; Yao et al., 2006), β -actin mRNA translation is specifically required for growth cone attraction (Leung et al., 2006; Yao et al., 2006). In addition, netrin-1 expressed in the tectum induces opposite effects on retinal axon branching that correlate with developmental stage and degree of axon differentiation (Shirkey et al., 2012). These findings suggest that alternative signalling pathways may be activated in growth cones to effect different responses to guidance cues depending on intrinsic factors and the environment. Furthermore, alternative responses may reflect the bifunctional roles of specific guidance cues in pathfinding and target innervation, providing a basis for the requirement of Vg1RBP in branching but not long-range navigation. While the *in vitro* turning assay is routinely used as evidence for the chemotactic function of guidance molecules, turning responses of cultured growth cones should be critically interpreted. BDNF induces growth cone turning which is dependent on β -actin mRNA translation (Leung et al., 2006; Yao et al., 2006), yet *in vivo* studies indicate that BDNF is involved in target-innervation but not long-range navigation (Cohen-Cory, 1999; Guthrie, 2007; Cohen-Cory et al., 2010). Thus in some cases, guidance molecules without chemotactic roles *in vivo* may activate turning responses when presented to growth cones in the simplified *in vitro* environment. There are other examples of signalling components, such as ubiquitin proteasome system-dependent protein degradation, that are required for growth cone turning *in vitro* (Campbell and Holt, 2001), but which affect branching and not long-range navigation when inhibited *in vivo* (Drinjakovic et al., 2010). We postulate that Vg1RBP-

dependent turning responses in cultured growth cones may reflect a role in signalling pathways mediating axon branching, rather than long-range pathfinding. One intriguing possibility is that netrin-1 can induce protein synthesis (PS)-dependent and -independent responses in growth cones, as has been found with different concentrations of Sema3A (Manns et al., 2012; Nedelec et al., 2012), and that only terminal branching is PS-dependent.

The growth cone is the critical subcellular compartment mediating axon extension and guidance. In contrast to the formation of filopodia-like protrusions extending from the axon shaft in the tectum, our results showed that the formation of growth cone filopodia and growth cone extension are not dependent on Vg1RBP or protein synthesis, consistent with previous findings. It has been reported that neither general protein synthesis inhibitors, β -actin mRNA translation, nor ZBP1 function, affect axon extension rates *in vitro* (Eng et al., 1999; Campbell and Holt, 2001; Leung et al., 2006; Sasaki et al., 2010; Welshhans and Bassell, 2012). No morphological defects have been reported in ZBP1-deficient growth cones, with the exception of a small but significant reduction in the length of growth cone filopodia (Welshhans and Bassell, 2012). Furthermore, neurons from CNS-specific β -actin knockout mice exhibit normal morphology *in vitro* (Cheever et al., 2012). These observations suggest that Vg1RBP or ZBP1 are unlikely to play a major role in growth cone morphology or motility, consistent with our finding that Vg1RBP is not required for long-range axon navigation. This is supported by a recent study showing that axonally synthesised β -actin promotes axon branching but not axon elongation *in vitro* or *in vivo* (Donnelly et al., 2013). It also suggests that the formation of filopodia in the growth cone and in the axon shaft involve distinct mechanisms that differentially require regulation of mRNA translation by Vg1RBP. However, some exceptions have been reported. Morpholinos against the β -actin mRNA zipcode have been shown to impair the motility of cultured chick forebrain growth cones (Zhang et al., 2001), and neurites of rat hippocampal neurons expressing non-phosphorylatable ZBP1 have a reduced length *in vitro*, suggesting impaired outgrowth (Huttelmaier et al., 2005). In addition, DRG neurons expressing a competitive exogenous β -actin 3'UTR that depletes axonal β -actin mRNA have reduced axon length *in vitro*, which is rescued by ZBP1 overexpression (Donnelly et al., 2011). Different culture conditions, species or cell-type specific differences could potentially underlie these discrepancies.

How does Vg1RBP promote *de novo* formation of axonal filopodia? It has previously been shown that BDNF-induced formation of dendritic filopodia-like protrusions in cultured mammalian neurons requires ZBP1 (Eom et al., 2003). Depolarization induces transport of ZBP1 to dendrites (Tiruchinapalli et al., 2003), and ZBP1 and β -actin mRNA are enriched at dendritic branch points (Perycz et al., 2011). Furthermore, blockade of the interaction between ZBP1 and β -actin mRNA reduces dendritic arbour complexity in cultured neurons (Perycz et al., 2011). These findings suggest that, similar to the mechanism described for growth cone turning, ZBP1 may regulate the localisation and translation of β -actin mRNA at sites of dendritic filopodia formation. We postulate that Vg1RBP plays a similar role in axonal filopodia formation. Our and previous studies show that filopodia emerging from the axon shaft arise from foci of cytoskeleton reorganisation characterised by an accumulation of F-actin, that corresponds with phosphatidylinositol-3-kinase (PI3K) activity (Lau et al.,

1999; Ketschek and Gallo, 2011; Spillane et al., 2011). Furthermore, NGF-coated beads have been shown to induce the accumulation of β -actin mRNA and filopodia formation at points of contact with the axon shaft (Willis et al., 2007). Although the present study focuses on β -actin, it is likely that Vg1RBP regulates the translation of numerous mRNAs at the site of filopodia formation. Growth factors that stimulate axonal filopodia formation have been shown to induce the localisation or local translation of various mRNAs including cortactin (Spillane et al., 2012), Par3 (Hengst et al., 2009) and peripherin (Willis et al., 2007). Vg1RBP binds mRNAs encoding cytoskeletal regulators including tau (Litman et al., 1996) and cofilin (Piper et al., 2006), but the complete repertoire of Vg1RBP targets has yet to be identified. We propose that Vg1RBP is at least one RNA-binding protein that regulates local mRNA translation at sites of axonal filopodia formation. Although we were unable to test the requirement for Vg1RBP granule localisation in filopodia emergence due to technical limitations of granule detection, we did observe examples of Vg1RBP granules localising to regions of the axon shaft just prior to filopodia emergence.

Our findings suggest that Vg1RBP promotes the emergence of axonal filopodia and branch formation by regulating the *local* synthesis of β -actin, and/or other proteins. In agreement, the axon branching defect described here resembles that observed in axons with depleted β -actin mRNA, which is rescued by axonally-targeted but not soma-targeted β -actin mRNA (Donnelly et al., 2013). However, the possibility that disrupted function of Vg1RBP in the cell body contributed to the observed branching defect cannot be excluded. For example, the recognition step of mRNA by RNA-binding proteins is critical to forming a transport RNP (Kress et al., 2004), therefore, loss of Vg1RBP could have broad effects in the cell body due to dysregulation of RNP complexes. Nonetheless, the fast timescale of events and the highly localized nature of axon-target contact sites that drive arbor formation are consistent with the involvement of a local mechanism of control.

Acknowledgements

We thank Nikki Coutts for performing cloning and transcribing DNA. We are grateful to Nancy Standart, University of Cambridge, for generously providing the Vg1RBP antibody. The work was funded by a Wellcome Trust Programme Grant (to CEH; 085314).

REFERENCES

- Campbell DS, Holt CE. Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron*. 2001; 32:1013–1026. [PubMed: 11754834]
- Cheever TR, Li B, Ervasti JM. Restricted morphological and behavioral abnormalities following ablation of beta-actin in the brain. *PLoS One*. 2012; 7:e32970. [PubMed: 22403730]
- Cohen-Cory S. BDNF modulates, but does not mediate, activity-dependent branching and remodeling of optic axon arbors in vivo. *J Neurosci*. 1999; 19:9996–10003. [PubMed: 10559407]
- Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. *Dev Neurobiol*. 2010; 70:271–288. [PubMed: 20186709]
- de la Torre JR, Hopker VH, Ming GL, Poo MM, Tessier-Lavigne M, Hemmati-Brivanlou A, Holt CE. Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. *Neuron*. 1997; 19:1211–1224. [PubMed: 9427245]

- Deiner MS, Kennedy TE, Fazeli A, Serafini T, Tessier-Lavigne M, Sretavan DW. Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. *Neuron*. 1997; 19:575–589. [PubMed: 9331350]
- Donnelly CJ, Park M, Spillane M, Yoo S, Pacheco A, Gomes C, Vuppalandhi D, McDonald M, Kim HH, Merianda TT, Gallo G, Twiss JL. Axonally synthesized beta-actin and GAP-43 proteins support distinct modes of axonal growth. *J Neurosci*. 2013; 33:3311–3322. [PubMed: 23426659]
- Donnelly CJ, Willis DE, Xu M, Tep C, Jiang C, Yoo S, Schanen NC, Kim-Safran CB, van Minnen J, English A, Yoon SO, Bassell GJ, Twiss JL. Limited availability of ZBP1 restricts axonal mRNA localization and nerve regeneration capacity. *Embo J*. 2011; 30:4665–4677. [PubMed: 21964071]
- Drinjakovic J, Jung H, Campbell DS, Strohlic L, Dwivedy A, Holt CE. E3 ligase Nedd4 promotes axon branching by downregulating PTEN. *Neuron*. 2010; 65:341–357. [PubMed: 20159448]
- Eng H, Lund K, Campenot RB. Synthesis of beta-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures. *J Neurosci*. 1999; 19:1–9. [PubMed: 9870932]
- Eom T, Antar LN, Singer RH, Bassell GJ. Localization of a beta-actin messenger ribonucleoprotein complex with zipcode-binding protein modulates the density of dendritic filopodia and filopodial synapses. *J Neurosci*. 2003; 23:10433–10444. [PubMed: 14614102]
- Falk J, Drinjakovic J, Leung KM, Dwivedy A, Regan AG, Piper M, Holt CE. Electroporation of cDNA/Morpholinos to targeted areas of embryonic CNS in *Xenopus*. *BMC Dev Biol*. 2007; 7:107. [PubMed: 17900342]
- Git A, Standart N. The KH domains of *Xenopus* Vg1RBP mediate RNA binding and self-association. *Rna*. 2002; 8:1319–1333. [PubMed: 12403469]
- Gumy LF, Yeo GS, Tung YC, Zivraj KH, Willis D, Coppola G, Lam BY, Twiss JL, Holt CE, Fawcett JW. Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization. *Rna*. 2011; 17:85–98. [PubMed: 21098654]
- Guthrie S. Neurotrophic factors: are they axon guidance molecules? *Adv Exp Med Biol*. 2007; 621:81–94. [PubMed: 18269212]
- Harris WA, Holt CE, Bonhoeffer F. Retinal axons with and without their somata, growing to and arborizing in the tectum of *Xenopus* embryos: a time-lapse video study of single fibres in vivo. *Development*. 1987; 101:123–133. [PubMed: 3449363]
- Hengst U, Deglincerti A, Kim HJ, Jeon NL, Jaffrey SR. Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. *Nat Cell Biol*. 2009; 11:1024–1030. [PubMed: 19620967]
- Hopker VH, Shewan D, Tessier-Lavigne M, Poo M, Holt C. Growth-cone attraction to netrin-1 is converted to repulsion by laminin-1. *Nature*. 1999; 401:69–73. [PubMed: 10485706]
- Huttelmaier S, Zenklusen D, Lederer M, Dichtenberg J, Lorenz M, Meng X, Bassell GJ, Condeelis J, Singer RH. Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature*. 2005; 438:512–515. [PubMed: 16306994]
- Ketschek A, Gallo G. Nerve growth factor induces axonal filopodia through localized microdomains of phosphoinositide 3-kinase activity that drive the formation of cytoskeletal precursors to filopodia. *J Neurosci*. 2011; 30:12185–12197. [PubMed: 20826681]
- Kislauskis EH, Zhu X, Singer RH. Sequences responsible for intracellular localization of beta-actin messenger RNA also affect cell phenotype. *J Cell Biol*. 1994; 127:441–451. [PubMed: 7929587]
- Kress TL, Yoon YJ, Mowry KL. Nuclear RNP complex assembly initiates cytoplasmic RNA localization. *J Cell Biol*. 2004; 165:203–211. [PubMed: 15096527]
- Lau PM, Zucker RS, Bentley D. Induction of filopodia by direct local elevation of intracellular calcium ion concentration. *J Cell Biol*. 1999; 145:1265–1275. [PubMed: 10366598]
- Leung KM, van Horck FP, Lin AC, Allison R, Standart N, Holt CE. Asymmetrical beta-actin mRNA translation in growth cones mediates attractive turning to netrin-1. *Nat Neurosci*. 2006; 9:1247–1256. [PubMed: 16980963]
- Li C, Bassell GJ, Sasaki Y. Fragile X Mental Retardation Protein is Involved in Protein Synthesis-Dependent Collapse of Growth Cones Induced by Semaphorin-3A. *Front Neural Circuits*. 2009; 3:11. [PubMed: 19826618]

- Litman P, Behar L, Elisha Z, Yisraeli JK, Ginzburg I. Exogenous tau RNA is localized in oocytes: possible evidence for evolutionary conservation of localization mechanisms. *Dev Biol.* 1996; 176:86–94. [PubMed: 8654897]
- Manitt C, Nikolakopoulou AM, Almario DR, Nguyen SA, Cohen-Cory S. Netrin participates in the development of retinotectal synaptic connectivity by modulating axon arborization and synapse formation in the developing brain. *J Neurosci.* 2009; 29:11065–11077. [PubMed: 19741113]
- Manns RP, Cook GM, Holt CE, Keynes RJ. Differing semaphorin 3A concentrations trigger distinct signaling mechanisms in growth cone collapse. *J Neurosci.* 2012; 32:8554–8559. [PubMed: 22723695]
- Nedelec S, Peljto M, Shi P, Amoroso MW, Kam LC, Wichterle H. Concentration-dependent requirement for local protein synthesis in motor neuron subtype-specific response to axon guidance cues. *J Neurosci.* 2012; 32:1496–1506. [PubMed: 22279234]
- Nieuwkoop, PD.; Faber, J. Normal Table of *Xenopus laevis*. Daudin; North-Holland; Amsterdam: 1967.
- Oberman F, Rand K, Maizels Y, Rubinstein AM, Yisraeli JK. VICKZ proteins mediate cell migration via their RNA binding activity. *Rna.* 2007; 13:1558–1569. [PubMed: 17652133]
- Percy M, Urbanska AS, Krawczyk PS, Parobczak K, Jaworski J. Zipcode binding protein 1 regulates the development of dendritic arbors in hippocampal neurons. *J Neurosci.* 2011; 31:5271–5285. [PubMed: 21471362]
- Piper M, Anderson R, Dwivedy A, Weinl C, van Horck F, Leung KM, Cogill E, Holt C. Signaling mechanisms underlying Slit2-induced collapse of *Xenopus* retinal growth cones. *Neuron.* 2006; 49:215–228. [PubMed: 16423696]
- Ross AF, Oleynikov Y, Kislauskis EH, Taneja KL, Singer RH. Characterization of a beta-actin mRNA zipcode-binding protein. *Mol Cell Biol.* 1997; 17:2158–2165. [PubMed: 9121465]
- Sasaki Y, Welshhans K, Wen Z, Yao J, Xu M, Goshima Y, Zheng JQ, Bassell GJ. Phosphorylation of zipcode binding protein 1 is required for brain-derived neurotrophic factor signaling of local beta-actin synthesis and growth cone turning. *J Neurosci.* 2010; 30:9349–9358. [PubMed: 20631164]
- Shewan D, Dwivedy A, Anderson R, Holt CE. Age-related changes underlie switch in netrin-1 responsiveness as growth cones advance along visual pathway. *Nat Neurosci.* 2002; 5:955–962. [PubMed: 12352982]
- Shirkey NJ, Manitt C, Zuniga L, Cohen-Cory S. Dynamic responses of *Xenopus* retinal ganglion cell axon growth cones to netrin-1 as they innervate their in vivo target. *Dev Neurobiol.* 2012
- Song HJ, Ming GL, Poo MM. cAMP-induced switching in turning direction of nerve growth cones. *Nature.* 1997; 388:275–279. [PubMed: 9230436]
- Spillane M, Ketschek A, Donnelly CJ, Pacheco A, Twiss JL, Gallo G. Nerve growth factor-induced formation of axonal filopodia and collateral branches involves the intra-axonal synthesis of regulators of the actin-nucleating Arp2/3 complex. *J Neurosci.* 2012; 32:17671–17689. [PubMed: 23223289]
- Spillane M, Ketschek A, Jones SL, Korobova F, Marsick B, Lanier L, Svitkina T, Gallo G. The actin nucleating Arp2/3 complex contributes to the formation of axonal filopodia and branches through the regulation of actin patch precursors to filopodia. *Dev Neurobiol.* 2011; 71:747–758. [PubMed: 21557512]
- Tiruchinapalli DM, Oleynikov Y, Kelic S, Shenoy SM, Hartley A, Stanton PK, Singer RH, Bassell GJ. Activity-dependent trafficking and dynamic localization of zipcode binding protein 1 and beta-actin mRNA in dendrites and spines of hippocampal neurons. *J Neurosci.* 2003; 23:3251–3261. [PubMed: 12716932]
- Tojima T, Hines JH, Henley JR, Kamiguchi H. Second messengers and membrane trafficking direct and organize growth cone steering. *Nat Rev Neurosci.* 2011; 12:191–203. [PubMed: 21386859]
- Welshhans K, Bassell GJ. Netrin-1-induced local beta-actin synthesis and growth cone guidance requires zipcode binding protein 1. *J Neurosci.* 2012; 31:9800–9813. [PubMed: 21734271]
- Willis DE, van Niekerk EA, Sasaki Y, Mesngon M, Merianda TT, Williams GG, Kendall M, Smith DS, Bassell GJ, Twiss JL. Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J Cell Biol.* 2007; 178:965–980. [PubMed: 17785519]

- Wu KY, Hengst U, Cox LJ, Macosko EZ, Jeromin A, Urquhart ER, Jaffrey SR. Local translation of RhoA regulates growth cone collapse. *Nature*. 2005; 436:1020–1024. [PubMed: 16107849]
- Yaniv K, Fainsod A, Kalcheim C, Yisraeli JK. The RNA-binding protein Vg1 RBP is required for cell migration during early neural development. *Development*. 2003; 130:5649–5661. [PubMed: 14522877]
- Yao J, Sasaki Y, Wen Z, Bassell GJ, Zheng JQ. An essential role for beta-actin mRNA localization and translation in Ca²⁺-dependent growth cone guidance. *Nat Neurosci*. 2006; 9:1265–1273. [PubMed: 16980965]
- Zhang HL, Eom T, Oleynikov Y, Shenoy SM, Liebelt DA, Dictenberg JB, Singer RH, Bassell GJ. Neurotrophin-induced transport of a beta-actin mRNP complex increases beta-actin levels and stimulates growth cone motility. *Neuron*. 2001; 31:261–275. [PubMed: 11502257]
- Zhang Q, Yaniv K, Oberman F, Wolke U, Git A, Fromer M, Taylor WL, Meyer D, Standart N, Raz E, Yisraeli JK. Vg1 RBP intracellular distribution and evolutionarily conserved expression at multiple stages during development. *Mech Dev*. 1999; 88:101–106. [PubMed: 10525193]
- Zivraj KH, Tung YC, Piper M, Gumy L, Fawcett JW, Yeo GS, Holt CE. Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. *J Neurosci*. 2010; 30:15464–15478. [PubMed: 21084603]

Figure 1.

Expression of Vg1RBP in *X. laevis* RGCs. A-F, Transverse sections of stage 33/34 (A-C) and stage 45 (D-F) eyes stained with an antibody against Vg1RBP and counterstained with DAPI. D'-F' show higher resolution images of the boxed regions in D-F. Scale bars, 100 μ m for A-F, 25 μ m for D'-F'. G, Western blot analysis using an antibody against Vg1RBP showing higher levels of Vg1RBP in lysates of stage 45 eyes compared to stage 33/34 eyes. H, I, Differential interference contrast (H) and fluorescence (I) images of a cultured axon immunostained with an antibody against Vg1RBP. Scale bar, 5 μ m.

Figure 2.

Dominant-negative Vg1RBP does not affect long-range navigation but increases axon overshooting. A. Schematic representation of Vg1RBP-eGFP and Δ KH4-eGFP showing the RNA-recognition motifs (RRM1/2) and KH didomains, indicating the deletion in the KH4 domain of Δ KH4-eGFP. B. Lateral view of mCherry-labelled axons expressing the indicated constructs, in the contralateral optic tract and tectum. Outlines of whole-mounted brains are indicated by dotted lines. Lower panel shows high resolution images of the boxed regions in the upper panel. Orientation of images is indicated in the cartoon on the left. Arrow,

overshooting axon. Scale bars, 100 μm in upper panel, 10 μm in lower panel. C-F, Histograms showing quantification of tract length, proportion of embryos with overshooting axons, overshoot length, and frequency of overshooting axons. Numbers in bars indicate number of embryos. $***P = 0.0004$, Chi-square test. G, Schematic representation of transverse view of the retinotectal tract (cartoon, left), and montages of images of serial sections of stage 41-42 embryos, showing gapGFP- and KH4-eGFP-expressing axons labelled with mCherry. Scale bar, 100 μm . Dor, dorsal; Ant, anterior; Tec, tectum; Ch, chiasm; ONH, optic nerve head.

Figure 3.

Quantification of mCherry co-expression with Vg1RBP-eGFP and KH4-eGFP in retinal neurons *in vivo*. A, Transverse section through a stage 42 eye co-electroporated with mCherry and KH4-eGFP, stained with DAPI (blue nuclear signal). Scale bar, 100 μ m. B-D, High resolution images of the boxed region in A. Scale bar, 10 μ m. E, Histogram showing high co-expression of mCherry with gapGFP, Vg1RBP-eGFP or KH4-eGFP, in retinal cell bodies. Numbers in bars indicate number of eyes analysed. F-K, Lateral view of stage 42 RGC axons and growth cones in the tectum, showing co-expression of mCherry and Vg1RBP-eGFP or KH4-eGFP. Scale bar, 5 μ m.

Figure 4.

KH4-eGFP blocks attractive growth cone turning in response to netrin-1 *in vitro*. A-D, Phase contrast images of control (A, B) and KH4-eGFP-expressing (C, D) growth cones at the start and 60 min after, exposure to a diffusible gradient of netrin-1. The position of the pipette ejecting netrin-1 is visible in the top right corner of each image. Scale bar, 20 μ m. E-G, Traces of the trajectory of growth cones during 60 min exposure to a netrin-1 gradient. The origin represents the position of the growth at the start of the experiment. Axes represent μ m. Netrin-1 stimulated a significantly different mean turning angle in control

($15.47^\circ \pm 5.68^\circ$; $P = 0.0159$, t test) but not KH4-eGFP-expressing growth cones ($-18.40^\circ \pm 4.77^\circ$; $P = 0.117$), compared with the mean turning angle of control growth cones stimulated with vehicle solution ($-5.75^\circ \pm 5.81^\circ$). H. Cumulative distribution histogram of the turning angles induced by netrin-1 and vehicle solution.

Figure 5.

Vg1RBP knockdown phenocopies the KH4-eGFP branching defects. A-B, Stage 39 embryos injected with 3 ng / blastomere CMO or AMO at 4-cell stage, showing reduced eye size in AMO-injected embryos. C, Western blot analysis of Vg1RBP levels in lysates of stage 33/34 eyes from CMO- and AMO-injected embryos. * $P < 0.05$, Bonferroni's post test. D-E, Transverse sections of stage 40 eyes from CMO- or AMO-injected embryos stained with an antibody against acetylated tubulin (red) and counterstained with DAPI (blue), showing normal retina organisation in AMO-injected embryos. D' and E' (scale bar 50 μ m).

show enlargements of the boxed regions in D and E (scale bar 100 μm), respectively. The optic nerve head (arrowheads) is thinner in AMO-injected embryos. F, Schematic representation of protocol for MO delivery and tract labelling. Dorsal blastomeres were injected with CMO or AMO at 4-cell stage, raised to stage 41-42 and fixed, then the left eye was injected with DiI to label RGC axons projecting to the contralateral tectum. G, Lateral view of DiI-labelled axons in the contralateral optic tract and tectum of CMO- and AMO-injected embryos. Outlines of whole-mounted brains are indicated in white dotted lines. Lower panel shows high resolution images of the boxed regions in the top panel. Arrowheads, axons of the retinofugal projection; arrows, overshooting axons. Scale bars, 100 μm for upper panel, 50 μm for lower panel. CTRL, control; Tec, tectum; Ch, chiasm; Dor, dorsal; Ant, anterior.

Figure 6.

Expression of Δ KH4-eGFP or Vg1RBP knockdown reduces retinal axon branching in the tectum. A, Lateral view of mCherry-labelled RGC axons in the tectum at stage 42. Indicated cDNA constructs were delivered by co-electroporation of the eye with mCherry. MOs were delivered by microinjection at 4-cell stage, followed by co-electroporation of eyes with mCherry. Scale bar, 10 μ m. Colour-coded traces indicate the main axon (black), primary (red), secondary (yellow), and tertiary (blue) branches. B-E, Histograms showing average number of branches per axon (B, D) and proportion of primary, secondary and tertiary axons (C, E). Numbers in bars indicate number of embryos.

Figure 7.

Time-lapse imaging of utrophin-mCherry (utr-mCh) and Vg1RBP-eGFP dynamics in branching RGC axons in the tectum. A, High resolution time-lapse image series of a segment of a gapGFP-labelled RGC axon, showing local accumulation of utr-mCh preceding the emergence of a filopodia-like protrusion. Arrowheads indicate the protrusion. B, High resolution time-lapse image series of a segment of an mCherry-labelled RGC axon, showing dynamic movements of Vg1RBP-eGFP granules during the emergence of filopodia-like protrusions. Arrowheads indicate emerging filopodia (two at $t = 0$ and a third at $t = 30$). In A and B, time is indicated in seconds, with $t = 0$ marking the emergence of a discernable protrusion.

Figure 8.

KH4-eGFP reduces the addition of axonal protrusions in RGC axons *in vivo*. A, High resolution time-lapse image series of a gapGFP-labelled RGC axon in the tectum over a 10 minute time-lapse imaging period using 30 second inter-frame intervals. Arrowheads indicate the emergence of filopodia-like protrusions. Scale bar, 5 μ m. B, Histogram showing the addition and removal rates of protrusions formed along the shaft of axons expressing gapGFP and KH4-eGFP.