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Coupling of NF-protocadherin signalling to axon guidance by cue-induced translation

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Abstract

Cell adhesion molecules and diffusible cues both regulate axon pathfinding, yet how these two modes of signalling interact is poorly understood. The homophilic cell adhesion molecule, NF-protocadherin (NFPC) is expressed in the mid-dorsal optic tract neuroepithelium and in the axons of developing retinal ganglion cells (RGC). Here we report that targeted disruption of NFPC function in RGC axons or the optic tract neuroepithelium results in unexpectedly localized pathfinding defects in the mid-optic tract. Sema3A, which lies adjacent to this turn, stimulates rapid protein synthesis-dependent increases in growth cone NFPC and its cofactor, TAF1, *in vitro*. *In vivo*, growth cones exhibit marked increases in NFPC translation reporter activity in this mid-optic tract region that are attenuated by blocking Neuropilin-1 function. Our results suggest that translation-linked coupling between regionally localised diffusible cues and cell adhesion can help axons navigate discrete segments of the pathway.

Introduction

Retinal ganglion cells (RGCs) send axons out of the retina, along the optic nerve, across the chiasm, and up the ventral optic tract (VOT). In the mid-optic tract (MOT), they change direction, turning caudally towards the tectum^{1, 2}. Localized expression domains of the repellent cues, Slit1, Slit2 and Sema3A, demarcate the rostral and dorsal boundaries of the optic tract and are thus in a good position to repel retinal axons from entering the telencephalon or extending too far dorsally³⁻⁵. Surprisingly, however, the combined knockdown of Sema3A and Slit1 causes axon stalling at the caudal turn in the MOT³, suggesting that such repellents may also somehow promote the next leg of the journey to the tectum.

Members of the cadherin superfamily of proteins play critical roles in establishing neuronal circuitry, particularly in axon initiation and synapse formation⁶⁻⁸. NF-protocadherin (NFPC) belongs to the non-clustered protocadherin sub-group of this superfamily and is the *Xenopus*

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Author Contributions L.C.L., C.E.H. and W.A.H. conceived the project, designed the experiments and wrote the manuscript. L.C.L. produced the constructs and peptides, generated and analyzed all the data in Figs 1-7 and Figs S1-7 unless otherwise stated. V.U. and T.G.B. performed and analyzed the *in vivo* Kaede translation reporter experiments in Fig. 8a,b. V.U. conducted the combined antibody *in vivo* Kaede reporter experiments in Fig. 8c,d and A.D. assisted with *in vivo* antibody experiments in Fig. S4e. M-L.B. performed *in situ* hybridization in Fig. S3. A.C.L. performed the Western blots in Fig. S1a,b and the cycloheximide experiments in Fig. S5n.

ortholog of Pcdh7. It consists of seven cadherin-like ectodomains, a single transmembrane domain and a C-terminal intracellular domain that binds its cytosolic partner, TAF1 (also known as TAF-I/SET). During embryogenesis, NFPC regulates ectodermal cell sorting and neural tube closure through homophilic adhesive interactions mediated by TAF1⁹⁻¹¹. NFPC is also strongly expressed in growing RGC axons and in the optic tract neuroepithelium during the development of the *Xenopus* retinotectal projection¹². In fact, the expression of NFPC in the neuroepithelium strikingly demarcates the mid-to-dorsal optic tract and precedes retinal axon entry into this region, suggesting that it may have a role in guiding axons through the caudal turn to the tectum. Interestingly, NFPC mRNA is expressed in RGC axons¹² raising the intriguing possibility that adhesive interactions can be translationally regulated by local cues that are known to elicit protein synthesis-dependent responses in growth cones *in vitro*¹³⁻¹⁷.

Here we show that targeted disruption of NFPC or TAF1 in either the axons or the neuroepithelial substrate leads to specific pathfinding defects in the MOT. The defects are similar to those seen with the combined knockdown of Sema3A and Slit1 (ref.3). Exploring this potential link, we find that Sema3A, rather than Slit, triggers a rise in NFPC in growth cones via local protein synthesis. Live imaging *in vivo* reveals that NFPC translation reporter activity shows a marked increase at the caudal turn that depends partly on Sema/Neuropilin signalling. Together the results show that NFPC-NFPC adhesion helps to guide axons at the caudal turn and suggest that cell adhesion can be spatially modulated by regionally expressed cues via a translation-linked mechanism.

Results

NFPC or TAF1 knockdown disrupts axon growth in optic tract

In a previous study, we found that expression of dominant negative NFPC and TAF1 constructs (NFPC Δ E and TAF1 Δ N) during early retinal differentiation inhibited axonogenesis in RGCs¹². To investigate the role of these molecules in axon pathfinding, we needed to inhibit their function in RGCs after axon initiation. We therefore used targeted electroporation¹⁸ to deliver NFPC Δ E-myc and TAF1 Δ N-myc into the retina at stage 32, when the first RGC axons have reached the optic chiasm¹⁹. Axon trajectories were analysed 24h later, at stage 40, when normal axons have completed their growth through the optic tract and reached the optic tectum (see schematic diagram in Fig. 1a). Compared to age-matched controls electroporated with an empty vector or GFP-only (Fig. 1b), many axons expressing NFPC Δ E-myc or TAF1 Δ N-myc stopped short of the tectum and exhibited abnormally high numbers of growth cones appearing to be stalled in the optic tract (Fig. 1c-e, Table S1 in Supplementary Information). Indeed, statistical analysis of axon length revealed that axons expressing NFPC Δ E-myc or TAF1 Δ N-myc were significantly retarded in their navigation compared to transfected controls (Fig. 1f).

We used antisense morpholino oligonucleotides (morpholinos) as an additional approach to knock down NFPC and TAF1 function^{10, 11}. Efficient knockdown, shown previously for these two morpholinos¹⁰, was validated here by Western blot analysis (Supplementary Fig. 1). The fluorescently-labeled morpholinos were electroporated into the retina at stage 32 (Fig. 2a',b',c') and the RGC axons were subsequently visualized by anterograde DiI labeling at stage 40. Axons from retinas loaded with control morpholino (Con-MO) showed a robust normal trajectory to the tectum by stage 40 (Fig. 2a) whereas axons from NFPC-MO-loaded eyes (Fig. 2b) were often short, and many appeared to stall or strayed anteriorly, particularly in the mid-optic tract segment around the caudal turn (Fig. 2b,d, Table S1). Similar axon phenotypes were also observed with the TAF1-MO (Fig. 2c,d, Table S1). The apparent discrepancy in the percentage of brains with axons entering the tectum with the two types of loss-of-function approaches (ca. 40% for dominant negative vs. ca. 95% for morpholino

knockdown; Fig. 1e and Fig. 2d) likely reflects the technical limitation that the fluorescent morpholino signal was too low to visualize in axons so all DiI-labelled axons were scored (including non-morpholino-loaded axons) whereas the myc-tag allowed identification and exclusive scoring of dominant negative-expressing axons.

These results suggest that axon defects caused by disrupting NFPC/TAF1 signalling occur predominantly around the mid-optic tract. To explore this further, we made time-lapse movies of control and dominant negative axons *in vivo* and compared the dynamics of their growth. Overall, NFΔE:eGFP-expressing axons advanced through the optic tract at a significantly slower average rate than GAP-GFP control axons (17.5μm/h vs. 26.6μm/h; $p=0.0336$, unpaired t-test, $n=21$; Fig. 3a,b, and Supplementary Videos 1,2). In addition, 30% of NFΔE:eGFP axons (7/23 axons) stalled at the caudal turn in the mid-optic tract, which was not observed in control samples (0/38 axons). A region-based analysis of growth (Fig. 3c) revealed a surprising subtlety in these phenotypes, namely that the speed of NFΔE:eGFP axon growth was normal through the ventral segment of the optic tract (VOT or “pre-turn”, 42.6μm/h vs. 38.5μm/h; Fig. 3d), but advancement in the dorsal tract after the caudal turn in the MOT (“post-turn”), if it occurred at all, was significantly retarded compared to controls (18.8μm/h vs. 30.7μm/h; Fig. 3d). Thus, axons expressing dominant negative NFPC grow at normal speeds through the VOT but slow down or stall at the caudal turn, supporting the idea that NFPC function is particularly important in this mid-to-dorsal segment of the optic tract.

Axon-substrate NFPC interactions required for navigation

To test whether NFPC cell-cell recognition is essential for retinal axon navigation in the dorsal optic tract, we designed a blocking peptide consisting of the ectodomain of NFPC. The NFPC ectodomain was fused to a Fc fragment, and this NFPC:Fc fragment was introduced into the intact optic pathway prior to the entry of retinal axons using the exposed brain preparation (see Methods and Supplementary Fig. 2a-d; ref. 1). Optic pathways were incubated for 12h in either NFPC:Fc or Fc tags alone (Con-Fc) and retinal axons were subsequently anterogradely labelled with DiI at stage 40. The majority of retinal axon trajectories from Con-Fc-treated brains appeared normal and axons entered the tectum (Fig. 4a-b). In contrast, the majority of NFPC:Fc-treated brains exhibited pathfinding defects in the mid-to-dorsal optic tract (Fig. 4c-e, Supplementary Fig. 2, Table S1). The defects included stalling at the caudal turn, and several misprojection phenotypes such as misrouting anteriorly into the telencephalon, bypassing the caudal turn, and failing to reach the tectum. Only 65.9% of NFPC:Fc brains had axons that entered the tectum compared to 100% of brains incubated with Con-Fc (Fig. 4e). Experiments to control for specificity showed that the treatments did not disrupt the expression pattern of the known key optic tract guidance cues, Slit1b and Sema3A (Supplementary Fig. 3).

NFPC is expressed in the mid-to-dorsal segment of the optic tract (Fig. 5a, and ref. 12), but the phenotypes seen with NFPC:Fc above do not distinguish whether the pathfinding defects are due to axon-axon or axon-substrate interactions. To test if NFPC function is required in the neuroepithelial cells of the optic pathway substrate, we disrupted NFPC function exclusively in the optic tract neuroepithelium by targeted delivery of NFPC and TAF1 morpholinos (NFPC-MO or TAF1-MO) into the cells of the presumptive optic pathway (diencephalon) at stage 29/30 by electroporation. At stage 40, the wildtype retinal axons were labelled with DiI to visualize their trajectories through the electroporated substrate. Morpholino-loaded areas of optic tract neuroepithelium were identified by green fluorescent signal. Electroporation of Con-MO into the optic pathway substrate did not affect the pathfinding of the retinal axons to the tectum (Fig. 5b). In contrast, NFPC- and TAF1-depleted substrate pathways exhibited a similar set of axon pathfinding errors as occurred with knockdown exclusively in retinal axons (Fig. 5c,d). When phenotypes were scored,

most of the retinal axons in Con-MO brains grew normally through morpholino-loaded areas (90.9%), whereas a large fraction of NFPC-MO or TAF1-MO brains (68% and 50%, respectively) had retinal axons that failed to grow through morpholino-loaded regions of the optic pathway (Fig. 5e, Table S1). Similarly, the proportion of brains in which retinal axons misproject out of the optic tract or bypass the caudal turn altogether (data not shown) is considerably higher in NFPC-MO (78.4%; $p < 0.001$, Fisher's exact test) and TAF1-MO (80.8%; $p < 0.001$, Fisher's exact test) compared to controls (4.5%). Finally, the proportion of brains that have retinal axons that stall at the caudal turn is significantly higher in NFPC-MO and TAF1-MO brains compared to the control condition (Fig. 5e). Together with the NFPC-Fc blocking-peptide results above, this suggests that NFPC-mediated interactions between retinal axons and their neuroepithelial substrate are crucial for pathfinding in the mid-to-dorsal optic tract.

Sema3A regulates NFPC and TAF1 in growth cones

Sema3A has been shown to be a repulsive guidance molecule for *Xenopus* retinal axons *in vitro* and *in vivo*^{3, 4}. Sema3A mRNA is expressed strongly in a domain adjacent and rostral to the mid-optic tract at the caudal turn (ref. 4 and Supplementary Fig. 4a), prompting us to ask whether Sema3A influences pathfinding of retinal axons *in vivo*. Blastomere injection (data not shown) and targeted forebrain electroporation of Sema3A morpholinos (Sema3A-MO), show that depletion of endogenous Sema3A (Supplementary Fig. 4b) leads to ectopic projections of retinal axons (Supplementary Fig. 4c). Indeed, 30% of Sema3A morphants showed at least one axon navigating away from the optic tract compared to 5% of Con-MO loaded samples (Supplementary Fig. 4d) and of the affected projections, approximately 10% of axons stalled at/near the caudal turn (Table S1). The relatively low penetrance may reflect a combination of mosaic MO expression, incomplete Sema3A knockdown and cue redundancy. We also used a function blocking antibody against neuropilin-1 (NP-1) as an alternative strategy to perturb Sema3A signalling *in vivo*. We first verified that this antibody abolishes the Sema3A-dependent collapse response in cultured retinal growth cones (data not shown), as previously seen⁴. The application of the antibody to exposed brain preparations caused misprojection of axons in the mid-optic tract in exposed brains *in vivo* (Supplementary Fig. 4e-f, Table S1), similar to Sema3A depletion. The broad similarity of Sema3A/NP-1 and NFPC stalling and misprojection phenotypes in the optic tract suggests that these molecules may work together to help guide retinal axons through the caudal turn and into the dorsal optic tract.

To explore the potential relationship between Sema3A and NFPC, we first tested whether Sema3A alters the level or the localization of NFPC in retinal growth cones *in vitro*. Consistent with previous observations⁴, the application of Sema3A caused significant growth cone collapse within 5 min, increasing to a maximal response of 60% at 10 min followed by substantial recovery by 60 min (data not shown). Notably, we found a rapid and dramatic increase in growth cone NFPC immunofluorescence following Sema3A exposure compared to control growth cones exposed to vehicle alone (Fig. 6a-b and Supplementary Fig. 5). This cue-induced NFPC response was specific to Sema3A, as it did not occur with Slit2 or LPA (Fig. 6c,d,f), two other cues which also cause retinal axon collapse within 10min (Fig. 6e, refs. 5, 13) This shows that the Sema3A-induced NFPC increase is cue-specific rather than a general feature of retinal growth cone collapse mechanisms. Upon stimulation with Sema3A, we observed NFPC in the filopodia (Fig. 6a and Supplementary Fig. 5h,i); such peripheral staining was rarely seen in non-stimulated growth cones (Fig. 6a and Supplementary Fig. 5b,c). By 60 min post-Sema3A exposure, when most axons had recovered from collapse, NFPC immunoreactivity had dropped (Supplementary Fig. 5m) and moved back into the central domain (Supplementary Fig. 5k,l). A similar Sema3A-induced increase and distribution of TAF1 was seen in these experiments (Supplementary

Fig. 6d,f). Interestingly, as seen previously²⁰, Neuropilin-1 (NP-1) showed the opposite change – a rapid down-regulation followed by recovery (Supplementary Fig. 6b,e,f), suggesting that NFPC is not acting in a receptor complex with NP-1 for Semaphorin 3A.

NFPC is translationally regulated by Semaphorin 3A/NP-1 signalling

Previous studies have demonstrated that Semaphorin 3A-induced growth cone repulsive responses can be blocked by inhibitors of protein synthesis and that Semaphorin 3A elicits rapid synthesis of new protein^{13, 14, 16, 21}. Since NFPC mRNA is found in retinal axons¹² and growth cones (Fig. 7a), we asked whether new protein synthesis contributes to the rapid rise of growth cone NFPC after 10 min. To test this, we performed 10 min Semaphorin 3A stimulations in the presence of transcriptional and translational inhibitors. Quantitative immunofluorescence analysis showed that the rapid increase in growth cone NFPC was completely blocked by anisomycin (Fig. 7b) or cycloheximide (Supplementary Fig. 5n), but not by inhibiting transcription with α -amanitin (Fig. 7b).

To investigate the dynamics of NFPC mRNA translation directly, we cloned the 3' untranslated region (3' UTR) of *Xenopus* NFPC and used it to drive expression of the photoconvertible protein Kaede (Kaede-NFPC-3' UTR; see Fig. 7c and Supplementary Fig. 7a). Photoconversion of Kaede from its native green to red in growth cones followed by cue-stimulation enables the live visualization of newly synthesized (green) proteins within minutes^{14, 22}. Our results showed that the Kaede-green signal increased progressively 10-30 min after Semaphorin 3A addition and Kaede photoconversion (Fig. 7c-d). The increase occurred in growth cones with intact axons (data not shown) but, importantly, was also significant in growth cones that were severed from their cell bodies indicating that new protein must be made locally within the detached process (Fig. 7c-d). Anisomycin blocked the increase and no significant changes in Kaede signal were detected without Semaphorin 3A stimulation (Fig. 7d) in vehicle-stimulated conditions during the 30 min period.

Since NFPC and Semaphorin 3A both act to promote the progression of axons past the caudal turn, we reasoned that Semaphorin 3A-induced translation of NFPC may occur *in vivo* in a region-specific manner, occurring preferentially in the mid-optic tract. To examine whether growth cones *in vivo* exhibit regional differences in NFPC translation dynamics, we expressed Kaede-NFPC-3' UTR in navigating retinal axons and monitored Kaede-green signal following photoconversion with timelapse microscopy at the ventral and mid-optic tract, i.e. either before or at the caudal turn (Fig. 8e). A significant increase in the native Kaede-green signal was detected in growth cones in the mid-optic tract, indicative of *de novo* synthesis of the reporter (5/10, Fig. 8b,f and Supplementary Video 4). In contrast, no increase in Kaede-green signal was detected in growth cones in the ventral optic tract (0/7, Fig. 8a,f and Supplementary Video 3). To test whether this region-specific increase in NFPC translation reporter activity is regulated by Semaphorin 3A, we used the NP-1 function blocking antibody *in vivo* to perturb the interaction of Semaphorin 3A with its receptor. When we applied the antibody *in vivo*, we observed a significant decrease in the levels of Kaede-green signal recovery following photoconversion in growth cones in the mid-optic tract (Fig. 8d, g and Supplementary Video 6) compared to a control antibody (Fig. 8c and Supplementary Video 5). Together, these data indicate that the local translation of NFPC in retinal growth cones is spatially regulated *in vivo*, by regionally expressed cues such as Semaphorin 3A, and suggest that cue-induced increase in homophilic adhesion is required for accurate pathfinding in the optic tract.

Discussion

Protocadherins have recently been implicated in the formation of major axon projections through regulating outgrowth^{12, 23}, dendritic arborisation^{24, 25}, spine number²⁶,

synaptogenesis^{27, 28} and neuronal survival²⁹. However, a direct role in axon steering has not previously been reported. Here, the observations that NFPC expression demarcates the early optic tract¹² and operates through a homophilic mode of signalling⁹, led us to investigate its role in establishing the optic tract. Using a combination of strategies to disrupt NFPC signalling *in vivo*, we found a striking regional requirement for homophilic NFPC function in the mid-dorsal optic tract, where retinal axons turn caudally toward the tectum. Pathfinding in this segment of the optic tract is known to require Sema3A³, and we provide *in vitro* and *in vivo* evidence that NFPC is rapidly upregulated in the growth cone by Sema3A through axonal translation, mediating homophilic NFPC interactions with the mid-to dorsal optic tract that support pathfinding in this segment of the pathway.

Cross talk between cell adhesion and Sema3A/NP-1 signalling was first described by Castellani and colleagues who showed that ligand binding induces direct associations between NP-1 and the classical cell adhesion molecule (CAM), L1, altering the Sema3A receptor complex itself, thereby modifying subsequent Sema3A-elicited axon behaviors^{30, 31}. Sema3A induces the endocytosis of L1/neuropilin complexes from the cell surface, leading to decreased axon-axon fasciculation, which is critical for the formation of the anterior commissure³¹⁻³³. A different semaphorin, Sema3D, promotes fasciculation by up-regulating L1 during the formation of the medial longitudinal fascicle axon tract in zebrafish³⁴. Our findings, however, point to a distinct mechanism operating for NFPC-Sema3A/NP-1 cross talk in retinal growth cones whereby Sema3A augments signalling through a non-classical cadherin, NFPC, via local translation. We have not tested whether the Sema3A receptor complex also participates in NFPC function, but this seems unlikely since Sema3A stimulates a simultaneous loss of NP-1 with NFPC increase. In contrast to a role in Sema3A signal transduction, our data instead suggest that NFPC translation in growth cones upregulates the abundance and peripheral localization of receptors to substrate-expressed NFPC required for guidance in this region. Intriguingly, previous studies have shown that substrate adhesion-based interactions can change the sensitivity of growing axons to semaphorin guidance cues. For example, a midline expressed cell adhesion molecule, NrCAM, mediates a Plexin-A1 switch in commissural axons that underlies their increased repulsive sensitivity to Sema3B³⁵ as well as differential responses to Sema6D³⁶. The interaction that we describe here works in the opposite direction with a localised guidance cue (Sema3A) affecting expression of a protocadherin, but it is an intriguing possibility that NFPC substrate interactions could feed back to adjust the growth cone's sensitivity to other cues at a subsequent choicepoint.

The finding that NFPC-depletion causes stalling in the mid-optic tract and slowed growth toward the tectum in the dorsal optic tract may seem puzzling given it is often assumed that an inverse relationship exists between the strength of adhesion and the speed of axon growth *in vitro*. However, while highly adhesive substrates do impede axon growth rates *in vitro*, the highest growth rates are observed at intermediate levels of adhesivity³⁷. In our live imaging studies, we note a trend that untreated retinal axons navigate slower in the dorsal optic tract than the ventral optic tract, which may result from the increased NFPC adhesion through substrate interactions. We also demonstrate that too little NFPC adhesion leads to difficulty navigating the caudal turn which is more in line with results in the grasshopper limb showing that homophilic cell adhesion, provided by the fasciclins, is crucial to prevent the stalling of axons at choice points³⁸. However, it is unclear whether NFPC, like fasciclins, also plays a major role in axon-axon fasciculation. We find that retinal axons stray from their normal trajectories when NFPC is depleted in the substrate alone prior to axon entry, demonstrating that NFPC axon-substrate interactions play a significant role in the formation of the optic tract. This does not exclude a role for fasciculation in optic tract guidance as increased interactions between axons destined for the tectum, through NFPC or otherwise, in concert with substrate recognition could also support correct pathfinding.

NFPC, like other protocadherins, mediates much weaker adhesion than catenin-binding classical cadherins⁹ and so is likely to be involved in different functions. Indeed, it has been shown that co-expressed cadherin subtypes have differing roles in supporting axon growth^{39, 40}. In addition, certain activity-dependent protocadherins also work heterophilically with classical cadherins as has been demonstrated with Arcadlin²⁶ and OL-protocadherin⁴¹. Thus, further studies are needed to understand how, in the growth cone, the plethora of adhesion molecules expressed with differential and combinatorial adhesion properties, combine to regulate the exact speed of axon growth *in vivo*.

Live imaging revealed that growth cones in the optic tract exhibit regional differences in the translation of a NFPC reporter. NFPC synthesis is induced by *Sema3A in vitro* and growth cones exhibit NP-1-dependent regional differences in NFPC translation reporter activity along the optic tract. In the NFPC-poor ventral optic tract where growth cones advance rapidly, the translation reporter activity is low. When growth cones reach the NFPC-rich mid-optic tract where they slow down and turn, NFPC translation reporter activity markedly increases. This region-specific dynamic regulation of NFPC is in line with previous studies showing that other transmembrane cue receptor molecules such as EphA2⁴² and Robo3⁴³ are up-regulated at specific locations in the pathway via local translation. Retinal growth cones (*Xenopus* and mouse) contain hundreds of different mRNAs⁴⁴ and specific guidance cues can trigger the translation of specific mRNA subsets. For example, the translation of β -actin mRNA in the growth cone is required for turning towards the attractant Netrin-1^{14, 17}, while repulsive Slit2 stimulation induces the synthesis of the actin depolymerising factor ADF/cofilin^{5, 15}. Similarly, NFPC mRNA is locally translated in response to *Sema3A*, but not Slit2. The NP-1-blocking antibody diminished but did not totally abolish the translation of Kaede-NFPC-3'UTR *in vivo*. This may be because the anti-NP-1 antibody is not capable of fully inhibiting the *Sema3A* in this assay, or because there may be other cues at the caudal turn that could also activate NFPC translation. mRNAs encoding several different protocadherins (e.g. protocadherins $\alpha 11$, β , $\gamma 3$, 9, 14) are also found in retinal growth cones⁴⁴, raising the possibility that their local synthesis may modulate other specific aspects of axon pathfinding and synapse formation.

TAF1 mRNA is also present in retinal growth cones⁴⁴ and the coordinate translation-dependent upregulation of TAF1 in response to *Sema3A* is consistent with its conserved role as a key effector of NFPC function¹⁰⁻¹². TAF1, first described as a transcriptional regulator⁴⁵, may have a role in the RGC nucleus prior to retinal axon initiation¹². However, since translation but not transcription is required for *Sema3A* chemotropic responses¹³, TAF1 is likely to have a non-transcriptional role at the growth cone in regulating NFPC adhesive signalling¹⁰.

Sema3A and Slit, classical axon repellents, are expressed in adjacent non-overlapping domains anterior to the developing optic tract near the caudal turn³⁻⁵. If these molecules acted solely as classical repellents, their depletion would be predicted to cause axons to stray into their normal domains of expression. Although some axons do exhibit these types of errors in *Sema3A*/Slit1-depleted brains, many axons stall unexpectedly at the caudal turn³. This stalling phenotype suggests that these molecules act not only as repellents but also give a "push from behind"³ to promote growth. But what is the nature of this 'push' and how does it facilitate axon growth beyond this point through the dorsal optic tract? Our results suggest that *Sema3A*, by upregulating axonal NFPC through local translation, helps promote axon growth in the NFPC-rich mid-to-dorsal segment of the optic tract, and thereby facilitates accurate navigation along the final segment of their route to the tectum. We suggest that the up-regulation of specific adhesion molecules in growth cones in response to encountering local diffusible cues could be used as a general mechanism to couple pathway experience to appropriate interactions for the journey ahead.

Materials and Methods

Xenopus embryos

Xenopus laevis embryos were acquired through *in vitro* fertilization as previously described¹², raised at 14–22°C in 0.1x modified Barth's saline (MBS; 0.88 mM NaCl, 0.01 mM KCl, 0.024 mM NaHCO₃, 0.1 mM HEPES, 8.2 μM MgSO₄, 3.3 μM Ca(NO₃)₂, 4.1 μM CaCl₂) and staged according to ref. 46. All animal experiments were approved by the University of Cambridge Local Ethical Review Committee.

DNA Plasmids

pCS2+ NFPC, NFΔE, TAF1, TAF1ΔN were gifts from Roger Bradley (Montana State University). “pCS2+ NFΔE:eGFP” construct was generated by amplifying eGFP from pN3:eGFP (Clontech) using the following primers containing 5' NcoI and 3' StuI restriction sites: eGFP-For: 5'-GCATGCCATGGGTAGTTCTGAATTCTGCAGTC-3' and eGFP-Rev: 5'-GCGAAGGCCTATTACTTGTACAGCTCGTC-3', and subsequently cloning the resulting eGFP fragment into pCS2+ NFΔE using the NcoI/StuI restriction sites 3' to the coding sequence.

“pFUSE NFPC Fc”: the 7 ectodomains of NFPC were amplified from pCS2+ NFPC using the following primers containing 5' EcoRI and 3' NcoI restriction sites: NFPC-For: 5'-GCGAATTCGATGATGAGGACTGAGGGGACAC-3' and NFPC-Rev: 5'-GCTCCATTGCTCTCTGCTTGCTCATGTATAGC-3', and ligated into pFUSE vector (InvivoGen) for use in COS-7 cell production.

“pCS2+ Kaede-NFPC-3' UTR”: as the published 3' UTR for NFPC was incomplete, a 3' RACE (Rapid Amplification of 3' ends) was performed based on manufacturer's protocol (Ambion) with modifications (see below and ref. 47). 1 μg of total RNA extracted from stage 40 *Xenopus* embryos was heated at 70°C for 5 min and chilled immediately on ice. Chilled components of a 3' RACE kit (Ambion First-Choice RLM-RACE kit) were added and incubated at 25°C for 5 min, 42°C for 60 min, 50°C for 10 min and inactivated with 15 min at 70°C. RNA was digested with RNaseH (Invitrogen) for 20 min at 37°C, leaving cDNA. Outer RACE reaction was then performed using outer RACE primer (Ambion), Outer NFPC forward primer (5'-TCACAGTGGAGGCTTCTAAG-3') and pFU polymerase and associated buffer (Stratagene). Following “touchdown” PCR (annealing temperature: 58.7°C), inner RACE reaction was performed as above using this time Inner RACE primer (Ambion) and Inner NFPC forward primer (5'-CTACTGCTGGCAAAAACAC-3'). Following gel purification, a standard “A-tailing” reaction was carried out using Taq Polymerase (Roche) (70°C for 30 min). The products were then ligated into pGEM-T easy vector (Promega) with DNA T4 ligase (NEB) (4°C overnight) to obtain pGEM-T-NFPC-3' UTR and the sequence of subcloned 3' UTR of NFPC was subsequently determined. Next, pGEM-T-NFPC-3' UTR was digested with MfeI followed by “Klenow” blunting, cut again with SacII and the NFPC-3' UTR fragment thus obtained. To finally generate pCS2+ Kaede-NFPC-3' UTR, the receiving vector pCS2+ β-actin 3' UTR kaede¹⁴ was double digested with SnaBI and SacII (to liberate the β-actin 3' UTR), then ligated with the NFPC 3' UTR insert with T4 DNA ligase. After sequence verification, capped mRNA was produced with SP6 mMessage mMachine Kit (Ambion).

Antisense morpholino oligonucleotides

Translation blocking morpholino oligonucleotides (MO, morpholinos) were synthesized by GeneTools using previously published sequences¹⁰. Morpholinos directed against *Xenopus* NFPC, TAF1 and Sema3A start sites (bold) were fluorescein tagged (3' end). Standard control morpholino, Con-MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3' (125 μM for

injection, 0.5mM-1mM for electroporations). NFPC-MO: 5'-TCTGTGTCCCCTCAGTCCTCATCAT-3' (125μM for eye electroporation, 0.5mM for tract electroporation). TAF1-MO: 5'-CATGTTGTGTGTGAGGAGAGGTGGT-3' (125μM for eye electroporation, 0.5mM for tract electroporation). Sema3A-MO A: 5'-TGCAATCCAGGTCAGAGAGCCCATG-3', Sema3A-MO B: 5'-GAGGTACAAGCAGATCCCTTAGCTC-3' (125μM for eye electroporation, 1mM for tract electroporation). Knockdown was verified by probing electroporated eye lysates with NFPC (1:500, gift from R. Bradley), TAF1 (1:400, Active Motif), Sema3A (brain lysate; 1:500, Abcam ab23393) antibodies using standard SDS-PAGE Western blot analysis.

Retina and brain-targeted electroporation

Electroporations of dominant negative constructs, morpholinos or RNA were carried out on stage 22-32 embryos as previously described¹⁸ using eight 18V pulses of 50ms duration, delivered at 1s intervals.

Production of NFPC ectodomains and exposed brain incubation experiments

COS-7 cells were grown in DMEM (Dulbecco's Modified Eagle Medium; Gibco) at 37°C until 80% confluency. To transfect the cells, fresh Lipofectamine (Gibco) was incubated in Opti-MEM (Gibco) for 30 min before 1-2μg of pFUSE-NFPC Fc or pFUSE-Fc (as the negative control: "Con-Fc") was added. After the cells were rinsed with Opti-MEM, 1mL of the transfection mixture was added and incubated for 5h at 37°C after which fresh medium was added. NFPC:Fc protein and Con-Fc protein was harvested after 5d by spinning down the cells and collecting supernatant in 1mL aliquots, which were snap-frozen and stored at -80°C. Before use, aliquots were thawed on ice and concentrated 20x with Centricon columns (YM30; Amicon). Some aliquots were separated by standard SDS-PAGE and probed for the Fc fragment to confirm chimaeric protein was made. When run alongside known concentrations of N-cad:Fc (R&D Systems), the amount of NFPC:Fc protein produced was estimated (see Supplementary Fig. 2a). For *in vivo* peptide/antibody blocking experiments, stage 33/34 exposed brain preparations (procedure described below in "*Live imaging of axon pathfinding*"; see Supplementary Fig. 2b-d) were incubated overnight in anaesthetic solution (0.4mM MS222 in 1.3x MBS) containing blocking peptides (see above, 1:100) or 0.5μg/μl anti-Neuropilin1 antibody (R&D, MAB566).

Live imaging of axon pathfinding

Live imaging of pathfinding retinal axons in the optic tract was performed as described⁴⁸. Stage 32 *Xenopus* embryos were transfected with either GAP-GFP or NFΔE:eGFP (see "*DNA plasmids*") and grown to stage 37. Embryos were selected based on good fluorescent signal in the eye and healthy appearance, and anaesthetized in MS222 in 1xMBS. Embryos were then restrained before skin and dura were carefully removed above the contralateral optic tract. They were next transected below the heart with a mounted razor blade and dissected heads were transferred to fresh 1.3xMBS containing 0.4mM MS222 before mounting in an imaging chamber consisting of two superimposed Gene Frame adhesive frames (Thermo) on oxygenated Permanox slides (Nunc). Images of axon pathfinding axons were taken every 3min for up to 24h per sample on an upright Nikon Eclipse 80i fluorescence microscope using Openlab acquisition software (Improvision). Axon growth was measured by tracing axon length from a user-defined fixed point and velocity was determined by subtracting overall growth of axons from their initial length over a defined period of time.

Fixation and Dil labelling of retinal axons

Embryos were fixed at appropriate stages with fresh 4% paraformaldehyde (PFA) in PBS overnight at 4°C. Dil injection into the retina was performed as described⁴⁹ using Dil crystals dissolved in ethanol to a concentration of 25mg/μL. After 20-24h in PBS at room temperature, brains were dissected and mounted in the lateral view to visualise optic tracts.

Analysis of fixed *in vivo* phenotypes

For dominant negative experiments, myc-positive axon lengths were traced and means were statistically compared. For Figures 1,2,4,5 and Supplementary Figure 4, the proportion of brains displaying axons projecting normally (i.e. reaching the tectum), stalling at the caudal turn or misprojecting was counted. Misprojections most frequently occurred near or at the caudal turn, but sometimes included other treatment-specific phenotypes such as axons that strayed ventrally into the telencephalon, axons that took aberrant dorsal trajectories and axons that avoided the tectum (see Supplementary Table 1). The percentage of axons that stalled or misprojected in the projections with aberrant phenotypes is presented in Supplementary Table 1.

In situ hybridization

pCS2-XSema3A (ORF of *Xenopus* Sema3A, subcloned in pCS2+ vector)⁴ and *pCMVSPORT6-xslit1* (Source Bioscience Lifesciences, image clone No 4930809) vectors were linearized and used as template for the production of digoxigenin (DIG)-labeled antisense riboprobes according to manufacturer's instructions (Roche). Whole brains or whole eyes were processed for ISH as described previously⁴. Probes were used at 2ng/μL. No signal was detected with sense probes (data not shown). Sample examination and photographs were taken with a Zeiss Axioskop microscope equipped with a Micropublisher 5.0 RTV camera. In situ hybridization on retinal growth cones was performed as described previously⁴⁴.

Retinal cultures

Retinal cultures were performed as previously described¹⁴. Eye primordia were dissected from anaesthetised animals, rinsed then plated onto glass coverslips/culture dish pre-coated with Poly-L-Lysine (10μg/mL; Sigma) and laminin (10μg/mL; Sigma). Cultures were incubated at 20°C for appropriate duration (typically 6-48h) before assays were performed on retinal axons.

Guidance cues and pharmacological reagents

Sema3A (R&D systems; 800ng/mL used for collapse and QIF experiments), LPA (1μM; Sigma) and Slit2CM (conditioned media courtesy of E. Hohenester) were used to stimulate retinal cultures in various assays (see below). 0.1% BSA in PBS, sterile double-distilled water and control conditioned medium were used as carrier controls for Sema3A, LPA and Slit2 respectively. For inhibitor assays, protein synthesis inhibitors anisomycin (40μM) or cycloheximide (50μM) or transcription inhibitor α-amanitin (10μg/mL; all from Sigma), were added to control and cue-stimulated cultures 1min prior to stimulation.

Growth cone collapse assay and quantitative immunofluorescence (QIF)

The growth cone collapse assay was carried out largely as described^{4, 50}. Retinal cultures were stimulated with appropriate cues (or carrier control) for 10min with or without pharmacological reagents before fixation (at least 15min) and either mounted in FluorSave (Calbiochem) or taken through immunocytochemistry (below). A growth cone was considered collapsed if it had fewer than two filopodia and one lamellipodium. Some of the above cultures were subsequently immunostained with standard immunocytochemical

protocols as described⁵ using antibodies against NFPC (1:500, gift from R. Bradley), TAF1 (1:400, Active Motif, 39202) and/or NP-1 (1:15, A5 clone, gift from H. Fujisawa). To quantify fluorescence resulting from antibody binding, fluorescence intensity was measured in Openlab software (Improvision) in randomly selected non-collapsed growth cones. Measurements were taken using masks obtained by manually tracing growth cone outlines in corresponding brightfield images, and background fluorescence, measured with the same mask for each growth cone, was subtracted from growth cone fluorescence. Immunofluorescence intensity was normalised to the mean immunofluorescence intensity of the control group.

Use of Kaede-NFPC-3'UTR translation reporter in cultured axons

Analysis of local translation of the Kaede reporter under the control of NFPC 3'UTR was performed as previously described for the β -actin 3'UTR^{14, 22}. Stage 32 *Xenopus* eyes expressing Kaede-NFPC-3'UTR, introduced by blastomere injection, were cultured overnight as described above. Retinal axons were isolated from their cell bodies by removing the explants with a mounted insect pin. Prior to Semaphorin 3A or control stimulation, Kaede-green in retinal axons was converted to Kaede-red by 8-10sec exposure to UV light as described previously²². A lower concentration of Semaphorin 3A (250ng/mL) was used compared to previous assays since the combination of the treatments in this assay was toxic to growth cones. For experiments requiring a block of translation, anisomycin (40 μ M) was added after Kaede photoconversion 1min prior to Semaphorin 3A. Images were acquired at 10 min intervals on a Nikon Eclipse TE2000-U inverted fluorescence microscope. To quantify fluorescence intensity, growth cone outlines were traced manually in Openlab (Improvision) and the change in fluorescence (F) from initial post-conversion fluorescence (F_0) was calculated ($F-F_0$). All measurements were made on growth cones with non-saturated signal and corrected for background fluorescence.

Use of Kaede-NFPC-3'UTR translation reporter *in vivo*

To image Kaede reporter translation *in vivo*, reporter mRNA was produced from the Kaede-NFPC-3'UTR construct by *in vitro* transcription using mMessage mMachine Kit (Ambion), purified using RNeasy Mini Kit (Qiagen), and electroporated into stage 26-30 eyes as described¹⁸. Stage 39 embryos were selected by green fluorescence in the eye and open brain preparations were prepared as described above. Kaede-expressing axons in the optic tract were visualized using UltraView VoX spinning disk confocal imaging system (Perkin Elmer) on an Olympus IX81 microscope. Kaede-green was photoconverted with a 10 sec pulse of 405nm laser illumination and visualized at 60x magnification using Volocity acquisition software (Improvision), capturing image stacks with 1 μ m distance between z-planes at 5 min intervals. To block NP-1 function *in vivo*, open brain preparations were incubated with function blocking anti-Neuropilin-1 antibody (0.5 μ g/ μ L; R&D, MAB566) or the corresponding IgG_{2B} control antibody (0.5 μ g/ μ L; R&D, MAB004) before photoconversion and imaging. To minimize potential diffusion of non-converted Kaede from the soma along the axon, these experiments were performed on growth cones of neurons lacking their cell bodies following the removal of the electroporated eye. For fluorescence quantification, growth cone outlines were traced manually in z-projections of multiple z-planes, using ImageJ (NIH). Fluorescence recovery was calculated from background-corrected intensity measurements by normalizing fluorescence change ($F-F_0$) to pre-conversion fluorescence ($(F-F_0)/F_p$).

Statistical analysis

Details of statistical analysis for each experiment are provided in figure legends or main text. Data were analysed in Prism (GraphPad). For all experiments, significance threshold of $\alpha=0.05$ was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Dominant negative NFPC signalling disrupts retinal axon growth and guidance in the optic tract

Retinal axons transfected with NFΔE-myc and TAF1ΔN-myc display defects in progression through the optic tract. **(a-b)** Stage 32 embryos electroporated in the eye (**b'**) with GFP to label retinal axons (asterisk) and resulting lateral optic tract at stage 40 as schematised (**a**) and shown (**b**) making the caudal turn (white arrow). **(c)** Co-transfection with NFΔE-myc in retinal axons, detected by myc staining (magenta) and merged with GFP channel (green), led to short axons (white arrows). 12μm myc-stained sections of electroporated eyes counterstained with DAPI (nuclei) show modest transfection of retinal cells (**c'**). **(d)** Expression of TAF1ΔN-myc in retinal axons also led to short axons (white arrows) from

modest transfection of retinal cells (d'). (e) Scoring of percentage of brains displaying aberrant phenotypes. Significant proportions of experimental brains have myc-axons that do not enter the tectum, and stall close to the caudal turn (* $p < 0.05$, *** $p < 0.001$, Fisher's exact test, number of brains in brackets). (f) Lengths of myc-positive axons were traced; NF Δ E and TAF1 Δ N axons were on average significantly shorter than pCS2+ empty controls (** $p < 0.001$, one-way ANOVA with Dunnett's post test). Lengths are expressed as percentages normalised to pCS2+ empty control group and mean brain length (represented in Fig. 1a), which did not differ significantly between groups ($p = 0.4929$, one-way ANOVA with Dunnett's post test). Number of axons measured shown within bars. Error bars = SEM. TEC, tectum; DOT, dorsal optic tract; MOT, mid-optic tract; VOT, ventral optic tract. Scale Bars: 150 μ m.

Figure 2. Depletion of NFPC or TAF1 disrupts retinal axon guidance in the optic tract

Loading of retinal axons with translation blocking morpholinos leads to aberrant pathfinding at the caudal turn. **(a-a')** Efficient loading of retinal cells with standard control morpholino (Con-MO), as shown in 12 μ m sections of electroporated eyes counterstained with DAPI (a'), reveal normal pathfinding in the optic tract (a; magnified inset shown in right panel). **(b-b')** Loading of retinas with morpholino against NFPC (NFPC-MO) leads to pathfinding defects at the caudal turn (b). Sections of electroporated eye counterstained with DAPI showed that lamination and size of retina remains unchanged (b'). **(c-c')** Loading of retina with morpholino against TAF1 (TAF1-MO) leads to aberrant pathfinding and stalling of

axons at the caudal turn as well as lower density of axons in the optic tract (c). Sections of electroporated eye counterstained with DAPI show that lamination and size of retina remains unchanged (c'). **(d)** Scoring of percentage of brains displaying aberrant phenotypes. While in all groups at least some DiI axons enter the tectum ("Axons project normally"), significant proportions of experimental brains have axons that stall and/or misproject close to the caudal turn (* $p < 0.05$, *** $p < 0.001$, Fisher's exact test, number of brains in parentheses). Error bars = SEM. Scale bars: a, b, c: 200 μm (left panels) or 100 μm in magnified insets (right panels); a', b', c': 150 μm .

Figure 3. Live imaging of retinal axons shows NFPC function is required at the caudal turn

Live imaging of NFΔE:eGFP axons show stalling and slower growth post caudal turn compared to GFP axons. **(a)** Time-lapse series showing the typical growth of an isolated GFP-labelled axon (arrows) over 135 min completing the caudal turn. **(b)** Time-lapse series showing the perturbed growth of an NFΔE:eGFP axon over the same 135 min period. **(c-d)** Post-hoc analysis was done measuring speed of growth through roughly defined regions of the optic tract pre- and post- caudal turn, which was based on the trajectory of the axon (c). NFΔE:eGFP axons were on average significantly slower in growing post-caudal turn while non-significantly different in other portions of the optic tract (d). ns: $p > 0.05$, * $p = 0.029$, one-way ANOVA. Number of axons shown in bold (in bars) with number of movies performed beneath in parentheses. Error bars = SEM. Scale bars: $5\mu\text{m}$.

Figure 4. Homophilic NFPC interactions are required at the caudal turn

Incubation of exposed brains with NFPC blocking peptide, NFPC:Fc, leads to aberrant pathfinding in the optic tract. **(a)** Inverse grayscale DiI images show normal pathfinding of retinal axons (black) when brains are incubated in the anaesthetic solution (adjacent panel is higher magnification of box in a). **(b)** Normal pathfinding is also observed when open brains were incubated in Fc only (Con-Fc). **(c-d)** Aberrant pathfinding particularly at the caudal turn is observed when brains are incubated with NFPC:Fc ranging from mild (c) to severe stalling phenotypes (d). There were also several other cases of retinal axons avoiding the tectum or misprojecting into telencephalon shown in Supplementary Fig. 2. **(e)** Graph displaying the proportion of incubated brains showing some axons with normal projections,

stalling or misprojection. The proportion of NFPC:Fc brains showing aberrant trajectories is significantly higher than controls in all categories. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Fisher's exact test (6 independent experiments, number of brains in parentheses). Error bars = SEM. Scale bars: left panels: 300 μm ; magnified insets (right): 150 μm .

Figure 5. NFPC is required in the neuroepithelium of the optic tract for guidance

Loading of the optic tract neuroepithelial substrate with translation blocking morpholinos leads to aberrant pathfinding at the caudal turn. **(a)** Wholemount *in situ* hybridisation of NFPC in stage 40 brain, neuroepithelial NFPC expression is particularly strong at the caudal turn (asterisk) and dorsal optic tract (DOT, black arrows) preceding axon growth into such areas. Dashed white lines demarcate the optic tract. **(b)** Brains electroporated with Con-MO show normal retinal axon trajectories labelled by DiI (red), with axons growing through morpholino loaded regions (cyan). Expanded image of DiI from inset is shown in right panel. Dotted lines demarcate outline of the brain (left) and optic tract (right). **(c)** Brains electroporated with NFPC-MO show aberrant pathfinding behaviors as well as stalling at the

caudal turn and misprojection anteriorly/ventrally into the telencephalon (white arrow). **(d)** Brains electroporated with TAF1-MO also lead to aberrant trajectories of retinal axons, showing stalled (arrow) and misprojecting (arrowhead) axons. **(e)** Graph showing the proportion of brains loaded with morpholino displaying phenotypes. A lower proportion of NFPC-MO (white bar) or TAF1-MO (grey bar) brains have axons growing through electroporated regions compared to the majority of Con-MO brains (black bar), which navigate normally. Conversely, the proportion of NFPC-MO/TAF1-MO brains showing aberrant phenotypes is significantly higher than Con-MO ones. $**p<0.01$, $***p<0.001$, Fisher's exact test (7 independent experiments, number of brains in parentheses). Error bars = SEM. TEC, tectum; DOT, dorsal optic tract; MOT, mid-optic tract; VOT, ventral optic tract; ch, optic chiasm. Scale bars: a and main panels in b-d: $300\mu\text{m}$; magnified insets in b-d: $50\mu\text{m}$.

Figure 6. Sema3A increases NFPC levels in retinal growth cones

(a-d) Immunostaining of cue-stimulated retinal growth cones shows rapid increase and redistribution of NFPC (heatmap, warmer colours indicate higher immunoreactivity) by Sema3A (b), but not Slit2 (c) or LPA (d). Morphology traces for growth cones (thin white dashed lines) were generated from respective phase contrast images (not shown). (e) Standard growth cone collapse assays show significant collapse (less than 2 filopodia and 1 lamellipodia) after 10 min stimulation by Sema3A, Slit2CM and LPA (* $p < 0.05$, Mann-Whitney U test). (f) Quantitative immunofluorescence (QIF) of non-collapsed growth cones (shown here) against NFPC show that within the induction of collapse, Sema3A significantly increases NFPC levels within 10 min, which did not occur with stimulation by Slit2 or LPA (** $p < 0.01$, Kruskal-Wallis test). Traces of non-collapsed growth cones were used to measure average fluorescence per unit area with background of substrate subtracted and normalised to vehicle control. Error bars = SEM. Scale bar: 5 μ m.

Figure 7. Sema3A induces local translation of NFPC mRNA

(a-a') Fluorescence in situ hybridisation (FISH) images showing positive NFPC signal with the NFPC antisense probe (a; green) and background signal with the sense control probe (a') in retinal growth cones (phase images combined with FISH). (b-d) Sema3A-induced rise in NFPC and Kaede-green signal are sensitive to translation inhibitors. (b) Quantitative immunofluorescence against NFPC showed that the rapid rise after 10 min Sema3A is blocked by the translation inhibitor anisomycin (40 μ M), but not the transcriptional inhibitor, α -Amanitin (** p <0.001, Kruskal-Wallis test). (c) Pre- and post-photoconversion images of isolated axons showing recovery of native Kaede-green, driven by 3'UTR of NFPC, in response to Sema3A stimulation. (d) Significant Sema3A induction of translation through the 3'UTR of NFPC in isolated axons is blocked by anisomycin (* p <0.05, Kruskal-Wallis test). Error bars = SEM. Number of growth cones assayed shown in key. Scale bars: 5 μ m. ANI, Anisomycin.

Figure 8. *In vivo* imaging shows NFPC-translation reporter activity is upregulated at the caudal turn and depends partly on NP-1/Sema signalling

(a) Growth cone expressing Kaede-NFPC-3'UTR in the Sema3A-poor ventral optic tract (VOT) showing efficient photoconversion of the native green Kaede signal to red. The absence of fluorescence recovery in the green channel over 30 min (arrowhead) indicates little or no *de novo* Kaede synthesis in the ventral tract. Dashed lines demarcate growth cone outlines. **(b)** Growth cones photoconverted as they navigate the caudal turn in the Sema3A-rich mid-optic tract (MOT) exhibit significant recovery of the green signal over the course of 30 min (arrowhead) indicating that growth cones actively translate NFPC in a region-specific manner. **(c)** Incubation of open brain preparations with a non-specific (IgG_{2B}) antibody does not affect the recovery of green signal in growth cones in the MOT (arrowhead). **(d)** Incubation with a function blocking Neuropilin-1 antibody (Anti-NP-1) reduces the recovery of the green signal in growth cones in the MOT (arrowhead). **(e)** Schematic diagram of sites of photoconversion and imaging of retinal growth cones. **(f)** The change in fluorescence from initial post-conversion level, normalized to pre-conversion

fluorescence. The green signal after photoconversion showed significant increase over time for growth cones in the MOT ($p=0.006$, Spearman $r=0.71$) but a decrease for growth cones in the VOT (ns, Spearman $r=-0.42$). **(g)** Incubation with anti-NP-1 causes a mild but significant decrease in Kaede-green recovery in the MOT compared to a nonspecific (IgG_{2B}) antibody ($p=0.009$, two-way repeated measures ANOVA). Error bars = SEM. Scale Bar: $5\mu\text{m}$.