A quantitative analysis of branching, growth cone turning and directed growth in zebrafish retinotectal axon guidance

Hugh D. Simpson¹, Elizabeth M. Kita¹, Ethan K. Scott², and Geoffrey J. Goodhill¹,³

¹Queensland Brain Institute, ²School of Biomedical Sciences, and ³School of Mathematics and Physics, The University of Queensland, Brisbane QLD 4072, Australia

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Corresponding Author
G. J. Goodhill
Queensland Brain Institute
The University of Queensland
Brisbane, 4072
Queensland, Australia
Phone: +61 7 334 66431
Fax: +61 7 3346 6301
Email: g.goodhill@uq.edu.au

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Abstract

The topographic projection from the eye to the tectum (amphibians and fish) / superior colliculus (birds and mammals) is a paradigm model system for studying mechanisms of neural wiring development. It has previously been proposed that retinal ganglion cell axons use distinct guidance strategies in fish versus mammals, with direct guidance to the tectal target zone in the former, and overshoot followed by biased branching towards the target zone in the latter. Here, we visualized individual retinal ganglion cell axons as they grew over the tectum in zebrafish for periods of 10-21 hours, and analyzed these results using an array of quantitative measures. We found that, while axons were generally guided directly towards their targets, this occurred without growth cone turning. Instead, axons branched dynamically and profusely throughout pathfinding, and successive branches oriented growth cone extension towards a target zone in a stepwise manner. These data suggest that the guidance strategies used between fish and mammals may be less distinct than previously thought.
1 Introduction

A paradigmatic example of a topographic map is the retinotectal (retinocollicular in mammals) map, in which neighboring retinal ganglion cells, representing neighboring points in visual space, connect to neighboring cells in the target region. Previous work has shown that competition, interactions among axons, gradient-based guidance cues, and correlated neural activity are all important in large scale map organization (reviewed in Goodhill and Richards, 1999; McLaughlin and O’Leary, 2005; Feldheim and O’Leary, 2010).

Studies of retinotectal / retinocollicular axon growth and targeting have been performed in a number of model systems, including fish (zebrafish, goldfish), amphibians (mainly *Xenopus*), birds (chicks), and mammals (rodents). These have led to the view that different classes of vertebrates use divergent mechanisms to form these maps (McLaughlin and O’Leary, 2005). In chicks and rodents, serial histology at different developmental timepoints has demonstrated that retinotectal / retinocollicular axons initially overshoot their appropriate rostrocaudal position, and then send out topographically appropriate interstitial branches towards their targets (Nakamura and O’Leary, 1989; Simon and O’Leary, 1992; Yates et al., 2001). Primary axon growth is generally straight and does not show evidence of turning, and branches tend to be at right angles to the axon shaft or parent branch. As the retina and superior colliculus / optic tectum of mice and chick do not grow significantly after the initial development of the retinotopic map, the locations of these initial arbors remains relatively stable.

However, the initial mapping in frogs and fish appeared different from the ‘overshoot and refine’ strategy used by birds and mammals. In *Xenopus*, retinal ganglion cells (RGCs) are born at stage 24 about a day after fertilization (staged according to Neiwooko and Faber (1994)), and within 6 to 8 hours RGCs extend axons that exit the eye, travel across the optic chiasm (stage 32) and enter the optic tectum at stage 37/38, forming the first synapses with tectal neurons at stage 40 (reviewed in McFarlane and Lom, 2012). The tectum continues to grow caudally, tripling in length between stages 42 and 50 (Sakaguchi and Murphey, 1985). The caudal growth causes the arbors to eventually occupy smaller percentages of the neuropil area (Sakaguchi and Murphey, 1985). In early stage 40 embryos, the arbors are already elaborate and have topographic organization, but this refines with tectal growth and becomes more precise as the embryo transitions from embryonic to larval stage 50 (Sakaguchi and Murphey, 1985).

A number of studies used still images to examine the morphology of axons in *Xenopus* embryos at stages 39/40 (Holt and Harris, 1983), stages 50 to 53 (Fujisawa, 1987), and stage 56 (Harris et al., 1987). Timelapse studies were also introduced to observe the behavior of axons traveling across the tectum. RGC axons from stage 35/36 and 40 embryos were visualized through DiI labelling and examined once a day for five days (O’Rourke and Fraser, 1990). In
a separate study, more frequent confocal images were taken during stages 45 to 46, with images every hour for six hours total (O’Rourke et al., 1994). Recently, the changes to branches and synapses observed in timelapse movies have been analyzed down to the level of electron microscopy (Li et al., 2011). Together, timelapse imaging studies in Xenopus have shown that retinotectal map formation results from directed axon growth, followed later by highly dynamic branch remodeling.

In zebrafish, similar results were reported, though based on fewer studies. In the zebrafish retina, the first RGCs are born between 29 and 34 hours post fertilization (hpf) (Moorman, 2001). RGC axons begin leaving the eye during the second day and begin to innervate their target areas at 3 days post fertilization (dpf) (Moorman, 2001). Through observations made in fixed animals spanning age ranges from 1.5 to 5 dpf, as well as in adults (Stuermer, 1988a) it was reported that retinotectal axons coursed directly to their targets, without significant branching en route. This view was supported by pioneering timelapse imaging studies in 2 to 5 dpf larvae (Kaethner and Stuermer, 1992). The temporal resolution was improved in a later study with growth cones and axonal arbors traced from images taken every 30 or 60 seconds during a span of 1 to 13 hours in 2 to 5 dpf embryos (Kaethner and Stuermer, 1994). These observations of ‘directed growth’, consistent with Xenopus provided a basis for claiming divergent mechanisms for the initial set up of topographic maps in mammals and chicks compared to fish and amphibians.

The eyes and optic tectum of fish continue to grow after the initial retinotectal map is established. New cells are added to the perimeter of the retina and the caudal domain of the tectum (Marcus et al., 1999). Because of this mismatch in location of new origin cells and new target destinations, the retinotectal map continues to shift during later stages of development, altering connections in order to maintain retinotopy. In particular, recent work has shown how guidance cues, neural activity, visual experience, and synaptogensis alter the dynamics of rearrangement in more mature zebrafish maps. This process has been observed over in a range of ages, including 3 to 5 dpf (Schmidt et al., 2000), 4 to 5 dpf (Hua et al., 2005), 3 to 4 and 9 to 10 dpf (Meyer and Smith, 2006), 3 to 5 dpf (Campbell et al., 2007), 7 dpf (Smear et al., 2007), 3 to 5 dpf (Leu and Schmidt, 2008), and 3 to 7 dpf (Fredj et al., 2010). The imaging itself is temporally restricted within the ages of embryo studied, ranging from two minute intervals between frames for durations lasting 40 to 50 minutes (Hua et al., 2005) to 10 minute intervals for durations of 10 hours (Meyer and Smith, 2006).

However, despite these recent careful analyses of post-arborization dynamics, understanding of earlier axon pathfinding across the zebrafish tectum is, in comparison, relatively limited. In particular, the dynamics of the early stages of pathfinding have not been examined in detail using more modern quantitative techniques. Thus, the directed growth mechanism
— the primary mechanism believed to be involved in fish retinotectal map development — is poorly understood. In particular, to grow directly to their targets, axons must somehow orient themselves to the correct bearing, but how axons achieve this, whether through turning, or other mechanisms, is unknown.
2 Materials and Methods

Breeding and raising

Adult zebrafish were obtained from the AZPF (Australian Zebrafish Phenomics Facility) at the University of Queensland, and kept on a 14 / 10 hour light / dark cycle. Timed matings were set up between heterozygous BGUG animals (male or female), and either wild-type or heterozygous Atoh7:GAL4 transgenic animals. Embryos were raised in E3 media with methylene blue (0.00003% or 30ppm) at 28.5°C. At the ages used, male and female larvae cannot be distinguished so we assume equal numbers of each were used.

Sparse labeling

Individual RGCs were visualized using zebrafish carrying the BGUG (Brn3c:GAL4;UAS:mGFP) transgene (Scott et al., 2007), which makes use of the yeast-derived GAL4/UAS system. The Brn3c gene is normally expressed in 40-50% of RGCs, and in these cells it drives UAS:mGFP in a variegated fashion so that only a few of the thousands of RGCs are labeled with mGFP. In some cases, BGUG was crossed to Atoh7:GAL4 transgenic fish to boost the frequency of RGC labeling.

Preparation of embryos

At 23 hours post fertilization (hpf), 200µM (0.003%) PTU (N-phenylthiourea, Aldrich) was added to the media to inhibit pigment formation and ensure animals were transparent during imaging (Karlsson et al., 2001). Animals were screened at 30 - 36 hpf for the presence of the BGUG transgene, evidenced by GFP expression in the ears (Xiao et al., 2005), and then again at 48 - 50 hpf for presence and where possible, number, of RGC axons in the optic chiasm or approaching the tectum. Animals with desired expression levels were then prepared for timelapse imaging.

Embryos were dechorionated at 48 - 50 hpf, allowed to rest for 30 min, and then immersed in clean E3 media containing 0.016% MS-222 (Sigma) for 15 - 30 min (ZFIN Zebrafish Book, http://zfin.org/zf_info/zfbook/zfbk.html). Embryos were then mounted ventral up for imaging in 1% low melting point (LMP) agarose (SeaPlaque Agarose, Lonza) containing 200 µM PTU.

All procedures were approved by the University of Queensland Animal Welfare Unit (approval SBMS/362/10/NHMRC).
In vivo imaging

Zebrafish embryos carrying the BGUG transgene were mounted at 48 - 50 hpf and imaged for 10 - 21hrs, beginning after axons were first seen approaching the tectum. Because of differences between the timing when a labelled axon first appeared in the imaging window, the movies span 48 - 96 hpf (2 to 4 dpf). Up to 10 embryos were mounted in a single 35 mm dish and imaged using a Plan-Apochromat 20x/0.8 M27 objective on a Zeiss LSM 510 inverted confocal microscope. The incubation chamber was set to 28.5°C. The microscope was equipped with a motorized stage and the Zeiss Multitime macro for imaging multiple positions. The 488 nm laser was at 7.5% power and we used a LP 505 filter to collect images. Image stacks approximately 40 to 50 µm in depth were recorded every 10 minutes, with each stack taking less than one minute. Master gain was set between 700 and 850 depending on the brightness of the axons, which varied from fish to fish. The pinhole diameter was 150 to 152 µm. We flattened the images into maximum intensity projections for analysis as axonal arbors are mostly planar (Fredj et al., 2010). Timelapse movies contained 60-150 frames. An example of several frames is shown as an image sequence in Fig. 1.

Whole embryo immunostaining

After the imaging window of 48 - 96 hpf (2 to 4 dpf), larvae were fixed, permeabilized, and immunostained. This permitted more accurate localization of axons on the tectum than using tectal autofluorescence during live imaging.

After the timelapse imaging, fish remained in their agarose for up to 12 hours. This was done for practical reasons based on the time the experiments ended (often late at night) and was typically only a few hours. Prior to fixation, fish were released from the chambers and allowed to recover, to ensure they were still vigorous and healthy. 4.5 to 6.5 dpf fish were anesthetized in 0.02% tricaine, then sacrificed by immersion in the fixative 4% PFA for 3hrs, washed out with phosphate buffered saline (PBS, pH 7.4), and stored in PBS with sodium azide. Larvae were later prepared for immunostaining by permeabilizing them in 1mg/mL collagenase for 1.5 - 2 hrs (depending on age at fixation) at room temperature, after which they were briefly post-fixed in 4% PFA (for 5 min). Primary antibodies to GFP and acetylated tubulin were applied at 1:1000 in PBS with 0.1% Triton X100 and left overnight. Primary antibodies were washed out with PBS/Triton and then secondary antibodies were applied at 1:1500 with 0.1% Triton and left overnight. Secondaries were washed out and the fixed and stained larvae were mounted in LMP agarose chambers in 35 mm Matek dishes similarly to the in vivo imaging, but this time in PBS with sodium azide (1% agarose for the beds, 0.5% to fill the chambers). Fixed, permeabilized, and stained animals were then imaged on the same microscope as was used.
for in vivo imaging. The Argon/2 488 nm laser was used at 30% power. The BP 505 - 550 filter was used with a pinhole diameter of 108 µm and a gain of 525 to image the green channel. The BP 575 - 615 IR filter was used with a pinhole diameter of 106 µm and a gain of 600 to acquire the red channel. To image the entire area, a z-stack 200 µm deep was used, with a 1.5x zoom through a Plan-Apochromat 10x/0.45 M27 objective. The resolution was 2048 x 2048 pixels. The images were edited slightly for brightness and contrast in Adobe Photoshop and labelled using Adobe Illustrator.

**Antibody Characterization**

The specifics of each antibody is listed in Table 1. In all cases, the antibodies produced staining patterns consistent with the expected results. Anti-GFP gave staining identical to, but brighter than, the endogenous mGFP. Anti-acetylated tubulin marked axonal tracts in a manner corresponding to publications from other labs (e.g. Fassier et al., 2010).

**Quantification and analysis of imaging**

Individual movie frames from timelapse imaging and images from fixed animals (Fig. 2) were both analyzed using ImageJ. Axons and arbors were traced using a customized version of the NeuronJ plugin, growth cones were tracked using the Manual Tracking plugin, and the resulting raw data were analyzed using custom Matlab programs. Where movement of the fish was a problem the Image Stabilizer plugin was used (Li, 2008). Using these tools, we were able to extract the following quantities from individual frames/images:

- Position, number, length, and order of branches.
- Branch endpoints & arbor center of mass
- Growth cone positions and velocities

Branch order classification was done by assigning order one to the primary axon, and order two to any branches off the primary axon, and so on until all branches were classified. To minimize direction bias, tracings were done blind to the final arbor position.

The timelapse imaging allowed us to examine how the above quantities evolved with time, and the images from fixed animals allowed us to confirm that the position of the arbor on the tectum was stable with time (for 1 - 3 days after the timelapse imaging). Hence we could analyze our quantitative data with regard to time and tectal positions of the RGC arbors. We were generally unable to obtain retinal soma positions for axons imaged. This was because for the majority of fish imaged, multiple axons were present, and fasciculation of axons in
retinofugal tracts meant we could not be sure which tectal arbor corresponded to which RGC soma.

Targeting behaviors were quantified spatially relative to the stable termination zone. The termination zone was taken as the position of the arbor in the final frame of each timelapse movie. We confirmed that this arbor position was relatively stable by cross-checking it with the position of the arbor in the fixed and stained images. The time of entry into the termination zone was defined as the time that an arbor area first includes the termination zone point, and stays there. The arbor area was defined using the convex hull around branch endpoints (see below), and it had to include the termination zone for at least 30 min (4 consecutive frames).

Branch point and growth cone classification criteria

To minimize subjectivity when tracing arbors and tracking growth cones, the following rules were adopted. A change in direction of an axon segment was considered more likely to be a branch point, rather than a bend or continuation, if there was: a change in angle of more than 30°, or a change of GFP signal intensity of more than 50%, or other branches from same point, or morphology like a branch point (’blob’ or knobby appearance), or there was previously a branch point at the same position. Based on this criteria, branches did not always end in a terminal tip. For example, In Fig. 2C, there are 18 branches but 16 free branch tips. Based on the rules above, the 90° bend in an upper-central branch describes the end of one branch and the start of a second. The other branch without a terminal tip comes from the end point of the primary axon shaft.

Motile processes on axons were classified as growth cones if they had morphology like a growth cone, moved a total of >10 µm, or existed for longer than 1hr. In tracing the ends of axons and branches that had a branched or stellate structure, it was not always possible to distinguish a growth cone with filopodia from a branch point with nascent branches. We traced all such significantly branched structures, noting that this could represent a true branch point or a skeletonized growth cone with filopodia.

To ensure reproducibility based on these rules, an additional independent observer traced one of the movies. While there were slight variations in the precise placing of branches between observers (mostly in the smaller, higher order branches), the branch ratios calculated from the two movies were not significantly different.

Analysis software

Custom analysis code was written in Matlab to process the raw arbor tracing and growth cone tracking data. For each quantity of interest, timeseries data were analyzed for individual axons /
growth cones, and also pooled to obtain group trends. Errors quoted are standard error unless otherwise stated.

Free branch endings, or terminal tips, were used for the analysis of the total area and centroid locations of each arbor. For all other analyses, every branch was counted, even those without terminal tips.

For the individual and grouped data, timescales were aligned by finding the time that an arbor arrived at its termination zone, setting this as time t=0, and aligning all axons/growth cones around this point. In the grouped data, time points around this alignment time contained data from all axons, while very early and very late time points contained data from fewer axons, due to differences in the lengths of timelapse recordings. Due to the variability in n-value at very early and late time points, we analyzed group data only when data from at least three axons were available for the aligned time point.

One movie (fish 1, axons 1-3) was recorded in 3 sections, of lengths 6hrs, 12hrs, and 3hrs. There were gaps between submovies of 1hr and 2hrs respectively. Data from submovies were combined for individual axon statistics, while the temporal structure of the submovies, including gaps, was preserved for timeseries plots of individual and grouped data.

**Branching dynamics.** We obtained instantaneous rates of branching, $\Delta N$, for each movie frame, by subtracting the number of branches $N$ in the previous frame $f-1$, $N_{f-1}$, from the number of branches in the current frame $f$, $N_f$; i.e. $\Delta N = N_f - N_{f-1}$.

We obtained an estimate of average rate of branch addition for each axon by summing all positive $\Delta N$ and dividing by the total movie time. Similarly, we obtained an average rate of branch loss by summing all negative $\Delta N$ and dividing by the total movie time. This method underestimates branch addition and loss because it does not individually identify branches.

We obtained improved estimates using a second method, where $\Delta N$ was calculated per branch order $o$, so that instead of a scalar, $\Delta N$ was a vector of length equal to the number of orders considered, and $\Delta \vec{N}_o = \vec{N}_{f,o} - \vec{N}_{f-1,o}$. Summing the positive elements of $\Delta \vec{N}_o$ gives an improved estimate of branches added from $f-1$ to $f$, and similarly summing the negative elements of $\Delta \vec{N}_o$ estimates the branches lost during the same interval. Average rates of branch addition and loss by order were calculated by again averaging over the length of the movie. Unless otherwise stated, values quoted for branching dynamics are calculated by order.

**Guidance measures.** We analyzed the mechanisms used by RGC axons to navigate towards their termination zone by looking at the motion of growth cones in relation to their target, and by looking at branching patterns relative to the target. To quantify branching patterns relative
to the termination zone, we calculated a branch ratio (BR) as a function of time:

$$BR(t) = \frac{B_T - B_A}{B_T + B_A}$$  \hspace{1cm} (1)

where $B_T$ is the number of branches directed towards the termination zone at time $t$, and $B_A$ is the number of branches directed away from the termination zone at time $t$. This measure takes on values from $-1$ to $1$, with positive values meaning an arbor has more branches directed towards, than away from its termination zone.

We measured growth cone turning by first calculating relative position vectors from one frame $f$ to the next frame $f + 1$. We then found the angle between this vector, and a vector from the position at frame $f$ to the position of the termination zone. In this way, for each movie frame we obtained the bearing $\theta$ relative to the eventual termination zone. By comparing subsequent angles, we could measure whether the growth cones’ bearings had turned towards or away from the termination zones. We used the average of this turning angle over the trajectory as one measure of turning. A positive average angle means that the growth cone, on average, turned towards the termination zone. A negative average angle means the growth cone, on average, turned away from the termination zone. Values around zero means there was no significant turning.

A second measure of turning we used was based on the above angle differences. Instead of using the actual angle values, for each frame we counted whether the turn was towards or away from the termination zone, and then calculated a turning ratio similarly to the branching ratio:

$$TR = \frac{T_T - T_A}{T_T + T_A}$$  \hspace{1cm} (2)

where now $T_T$ and $T_A$ are the total number of turns towards and away for a single trajectory, respectively. Note that this is calculated over an entire trajectory, and not for each frame, so it is not a function of time like the branch ratio.

**Growth and movement correction.** During 2 to 4 dpf time period, larvae continue to grow, and a slight increase in size can be observed during timelapse imaging. Additionally, the larvae straighten around the yolk sac, rotating the head from $140^\circ$ to $160^\circ$ relative to the tail axis (Kimmel et al., 1995). The design of the imaging chambers minimizes the changes to the viewing angle. Between larval growth and rotation, the average shift relative to the objective was $3.7\pm0.3\mu m/hr$. We accounted for this by using the movement of the position of the proximal primary axon as a reference, as this should not move with time, except for growth. Where multiple axons were traced in the same movie, movies where the location of the proximal axon could not be determined accurately were excluded, and the remaining movies were averaged.
to get a proximal axon position with time. The termination zone was then offset for each frame by the amount of growth remaining in each movie.
3 Results

We studied retinotectal axon growth and targeting by performing timelapse imaging of sparsely labeled zebrafish RGC axons. We measured growth cone turning, selective branching and 'directed growth', which we defined as target-directed growth cone motion. Timelapse videos of 10 axons from six embryos taken during the range of 48-96 hours post-fertilization (hpf) were included in the analysis, created from images taken every 10 minutes. Videos were only included in the analysis if one or more axons could be individually resolved, and imaged from tectal entry to target arrival. The advance across the tectum took between 10 and 21 hours, depending on final tectal position, giving 162 hours of timelapse video in total (Table 2). In Fig. 1, $t = 0$ represents the time an axon was first seen on the tectum. However, in all figures following, $t = 0$ was defined as the time of arrival at the axons’ termination zone, and grouped data were aligned at this arrival time.

3.1 Branch addition and loss are highly dynamic and balanced over time

We quantified branching dynamics of growing axonal arbors by finding average branching rates over the course of an axon’s growth, as well as instantaneous behavior at each time point (Fig. 3). Total number of branches increased steadily with time for all axons, both before and after arrival at the termination zone (Fig. 3A, E).

Branching rates were determined by comparing the number of branches added or subtracted from one frame to the next. We did this for all branches, and also by branch order (see Methods). Most axons added and lost no more than 10 branches every 10 minutes (e.g., Fig. 3A,B), although the more active axons added and subtracted up to 20 branches per frame. The tight balancing of branch addition and subtraction was evidenced by the large number of branches added and lost compared to the small number of net branches added (Fig. 3C,D). The same trend is seen in the remarkably small net branch difference averaged over all axons (Fig. 3F; Table 3). When grouped and compared over time, the branching dynamics did not suddenly increase in activity after axons arrived at the termination zone (Fig. 3G). Surprisingly, branch activity was high even before the axons arrived at their target, suggesting the potential involvement of branching in guidance to the target.

There was minimal variation in branching rates among axons, including when normalized to total arbor length, and rates of addition mirrored rates of loss (Fig. 3H; Table 3). Rates calculated by order were in all cases greater than rates calculated without regard to order (Fig. 3H). This reflects the notion that identifying branches by order, and comparing changes in number of branches of each order between frames, provides a better estimate of branching rates than simply comparing total branch number from frame to frame. For example, the addition of
a second order branch and the elimination of a third order branch are seen as two separate events using this technique, rather than cancelling each other out (see Methods). Of the hundreds of branches added and retracted during axonal growth towards the termination zone, only 5-10% of branches remained at the final points of the timeseries data, emphasizing the highly dynamic nature of branching during pathfinding behavior (Fig. 3F; Table 3). Averaged over all axons, the rate of branch addition was $0.145 \pm 0.013 \mu m^{-1}hr^{-1}$, the rate of branch loss was $0.104 \pm 0.007 \mu m^{-1}hr^{-1}$, and the two were significantly different (two-tailed t-test, $p < 10^{-10}$), consistent with net addition of branches over time.

### 3.2 Growth cone velocities remain steady during and after navigation to the target

We investigated growth cone dynamics by tracking individual growth cone positions in each frame of the timelapse recordings. Only growth cones that could be tracked for at least 6 frames (1hr), and moved in that time a total of at least $10 \mu m$ were included (40 growth cones in total).

Each axon typically had several growth cones meeting tracking criteria, and new trajectories were often bifurcations of previous ones (Fig. 4A). Instantaneous velocities of growth cones varied within trajectories, but the variation was consistent between growth cones (Fig. 4B). There were no obvious upward or downward trends in velocities over time when averaged for individual axons (Fig. 4C), or when averaged over all axons (Fig. 4F). Hence growth cone velocities, on average, remained roughly constant throughout the time that axons were imaged. This includes times after arrival at the axons’ target, where growth cone velocity did not drop to zero as might otherwise be expected. Growth cones were still active after arrival at the target area, and the velocity did not change as they transitioned from growth during navigation to local exploration within the target zone.

Velocities averaged over individual axons were $10.3 - 13.4 \mu m/hr$ (Fig. 4D, Table 4). Despite consistent averages, individual growth cones displayed a wide range of instantaneous velocities, with all growth cones remaining still at some points, and reaching speeds of up to $65 \mu m/hr$ (Fig. 4E), Table 4). Averaged over all growth cones, the mean velocity was $11.7 \pm 0.25 \mu m/hr$.

### 3.3 Axons are guided by a combination of selective branching and target-directed growth cone motion but not growth cone turning

Zebrafish retinotectal axons have been observed to grow directly to their targets (Kaethner and Stuermer, 1992), but how they become correctly oriented has not been clearly established.
Turning is an obvious candidate for this purpose, so we analyzed this first, using quantification of trajectory shape and direction, turning angles, and turning ratios (see Methods).

Growth cone trajectories generally appeared straight, rather than curved (Fig. 5A,B). 83% of trajectories (33/40) had a significant \( p < 0.05 \) linear correlation in their \( x \) and \( y \) positions, so that the majority of trajectories could be fit with a straight line. Angles turned by growth cones relative to their targets were computed by comparing bearings at subsequent time points. These instantaneous turning angles within trajectories were approximately zero, albeit with some variability (Fig. 5C). When averaged over all axons / growth cones at time points with \( N \geq 3 \), the turning angles at 94% (60/64) of the time points were indistinguishable from zero (Fig. 5D). When these angles were averaged over time for each growth cone, means for all 40 growth cones were statistically indistinguishable from zero in a t-test (Fig. 5E). The mean for all growth cones was \( \theta_{\text{diff}} = 0.7^\circ \pm 0.7^\circ \), and not significantly different from zero. Turning ratios compared the number of turns towards the target with the number of turns away from the target within trajectories, and these were also centred around zero (Fig. 5F), with a mean of \( TR = 0.001 \pm 0.03 \). Thus, surprisingly, there was no evidence of turning in the trajectories of growth cones analyzed.

If growth cones do not turn, do they show other forms of target-directed motion? Certainly the axon / arbor as a whole must gradually move towards its target, and when quantified as the distance of an arbor centroid to its termination zone through time (Fig. 6A), this was indeed the case. To assess whether growth cones themselves were undergoing any target-directed motion, we measured the distance from growth cones to their termination zones through time. Most growth cones studied were observed to move steadily and straight, with the overwhelming majority growing towards their targets, rather than away (Fig. 6B,E). To quantify this, we used the slope of the target approach curve for each trajectory (velocity relative to target). This slope, which we called the targeting gradient (TG), reflects whether a growth cone is growing towards its termination zone (negative gradient), away from its termination zone (positive gradient), or neither (approximately zero slope). We used this targeting gradient (units of \( \mu m/hr \)) as a measure of target-directed growth cone motion.

The targeting gradient showed strong evidence for target-directed growth cone motion, in that it was typically negative (a single axon example is shown in Fig. 6C, all growth cones plotted in Fig. 6F). When averaged over the time from tectal entry to arrival at the termination zone, the targeting gradient for 8/10 axons was negative, and significantly different from zero in a t-test (Fig. 6D). Timepoints were only analyzed when \( N \geq 3 \). From tectal entry to arrival at the termination zone 33% of timepoints (27/82) had targeting gradients significantly less than zero, only 1% (1/82) had targeting gradients significantly greater than zero, and 66% (54/82) were indistinguishable from zero in a t-test. During this period the average targeting gradient over
all axons / growth cones was $-0.58 \pm 0.06\mu m/hr$, demonstrating statistically significant target-directed growth during this time. After arrival at the target, the gradient was close to zero, reflecting growth cones moving both towards, away from, and sometimes around, the target at this time.

If growth cones do not turn, but nevertheless show a strong trend of growing towards their target, they must use another mechanism to orient to the correct bearing. During manual tracing of timelapse movies, striking asymmetries in branching patterns of arbors were observed (Fig. 7A - D). Sometimes only for a few frames ($<30$ min), and sometimes for longer periods (1 - 2 hrs), axons had more branches directed towards their targets than away. Based on this, we hypothesized that selective branch addition and loss could play an orienting role, similar to the biased interstitial branching observed in chicks and mammals. To quantify the presence of target-directed branching, we calculated a branch ratio as a function of time, for each axon (see Methods).

The branch ratio timeseries were predominantly positive, indicating more branches directed towards the termination zone than away, up until arrival at the termination zone (the timeseries for the axon in Fig. 7D is shown in Fig. 8A). This trend was reflected in the averaged data for all axons (Fig. 8C), with positive values shown for most of the timelapse imaging. Branch ratios always turned negative after arrival at the termination zone, because at this time branches will inevitably be directed away from the termination zone. Before this time, 24% of timepoints (19/80) showed a branch ratio significantly greater than zero, none showed a branch ratio significantly less than zero, and 76% (61/80) were not distinguishable from zero (based on t-tests at each timepoint). Grouped data were analyzed when $N \geq 3$. Averaged over the time from tectal entry to arrival at the termination zone, most axons (7/10) had positive branch ratios significantly different from zero in a t-test (Fig. 8B). Two axons had branch ratios statistically indistinguishable from zero, and one had a negative branch ratio that was significantly different from zero. The mean branch ratio for all axons measured up until arrival at the termination zone was $BR = 0.29 \pm 0.02$, showing a statistically significant trend from tectal entry to arrival at the termination zone. These results suggest that selective branching plays an important role in zebrafish retinotectal axon navigation.

In Fig. 8D we directly compare the branch ratio and targeting gradient measures of guidance. The two quantities are significantly negatively correlated (Pearson’s $r = -0.54$, $p = 10^{-10}$).

These data show that zebrafish RGC axons use both directed growth cone motion and selective branching to navigate to their targets. Turning does not appear to play a significant role in guidance, and instead axons use selective branching to orient themselves correctly, and directed motion of growth cones to approach their targets.
3.4 Distance to travel, but not retinotopic position, affects guidance and rates of branching

Growth cone velocities, axon branching rates, and measures of guidance showed variability from axon to axon. We hypothesized that this variability may be partly due to differing positions of axons within the topographic map. We therefore compared quantities of interest with target position along rostrocaudal and mediolateral tectal axes (Fig. 9A-G), which were measured using images from fixed animals at 4.5 to 6.5 dpf, and also with initial distance to target (Fig. 9H), computed from the timelapse data. As mentioned previously (see Methods) it was not possible to determine the retinal location of the cell body corresponding to an arbor in the tectum, and thus tectal location of the target zone was used as a surrogate measure.

There were no significant trends in variation of velocities with target rostrocaudal (RC) or mediolateral (ML) positions (Fig. 9A,B). There was a significant association between branching rates and rostrocaudal position (extension: \( r = -0.64, p = 0.047 \); retraction: \( r = -0.81, p = 0.004 \)) but not mediolateral position (Fig. 9C,D). Based on this it appears that axons with further to travel from their rostral tectal entry point exhibited lower overall rates of branching. This is consistent with the idea that these axons undergo longer periods of axonal growth without significant branching. Another potential reason for these trends is that branching may be inhibited in caudal tectum by gradients of ephrinA, as is the case for mammals (McLaughlin and O'Leary, 2005) and *Xenopus* (Woo et al., 2009). The mediolateral axis branching rates do not show a significant trend.

Guidance measures were averaged over all timepoints up until the point of arrival at the termination zone to obtain a single number for quantifying guidance for each axon. These values were then compared with stable rostrocaudal and mediolateral tectal position (Fig. 9E,F). There were no statistically significant trends for branch ratio or targeting gradient, compared with rostrocaudal map position (branch ratio: \( r = 0.54, p = 0.11 \); targeting gradient: \( r = 0.41, p = 0.24 \)), or mediolateral map position (branch ratio: \( r = 0.10, p = 0.78 \); targeting gradient: \( r = 0.22, p = 0.54 \)). Hence variables other than map position are required to explain the variance in the guidance mechanism data.

During data analysis we noticed that axons tended to enter the tectum via one of several fascicles located rostral to the tectum (see Fig. 2F), and that axons often moved laterally or medially after this. This suggested that axons might be correcting their course after initially entering in the wrong mediolateral position. Rough, but not perfect, mediolateral position on tectal/SC entry has been observed previously (Bunt and Horder, 1983; Lee et al., 2004; Plas et al., 2005). Hence we hypothesized that mediolateral difference between the tectal point of entry and the stable tectal position (\( \Delta \text{ML} \)) might explain variation in guidance measures (Fig. 29H).
Although there were weak correlations between $\Delta ML$ and both guidance measures, they were not significant (branch ratio: $r = 0.55$, $p = 0.1$; targeting gradient: $r = 0.59$, $p = 0.07$). Errors in mediolateral entry alone could not, therefore, adequately explain variation in guidance measures either.

A more general measure of the error correction required by navigating axons is the distance from the initial point of tectal entry to the eventual termination zone (determined from timelapse imaging, rather than images of fixed animals). When we compared this quantity to guidance measures for each axon, we found significant correlations between both branch ratio ($r = 0.83$, $p = 0.003$) and targeting gradient ($r = 0.77$, $p = 0.01$) (Fig. 9H). This result supports the idea that axons use both selective branching and directed growth cone motion in proportion to how far away from their termination zones the axons begin.
4 Discussion

Summary of live imaging results

We obtained quantitative data on the branches and growth cones of retinal ganglion cell axons during zebrafish development, focusing on 2 to 4 days post fertilization (dpf) when the initial retinotectal map is forming. Although several recent studies have overlapped this time frame, none have examined axon behavior in respect to navigation to the target location on the tectum. In addition to providing quantification of variables such as average velocities and branching rates, these data contribute to our understanding of the nature and dynamics of axon targeting and arbor refinement.

Axons enter the tectum with varying distances to travel to their termination zone (Fig. 9), and begin their navigation with few or no branches, consistent with what has previously been seen (Stuermer, 1988a; Kaethner and Stuermer, 1992, 1994). During 2 to 4 dpf, initial growth cone trajectories are often erroneous (Fig. 8D) and the axon enters a phase of exploratory growth. During this time the axon navigates using a combination of guidance by selective branch addition and subtraction (Fig. 7, Fig. 8), and directed motion of growth cones towards the target area (Fig. 6), but not growth cone turning (Fig. 5), with the extent of both types of guidance proportional to the initial distance to the termination zone (Fig. 9). During navigation there are high levels of branching, with branch addition and removal remaining tightly balanced at all times, such that only a small fraction of branches extended remain at any one time (Fig. 3, Table 3). This is consistent with the high turnover of branches others have observed at this developmental timepoint, i.e. in zebrafish as young as 3 to 4 dpf (Meyer and Smith, 2006), and slightly later, at 4 to 5 dpf (Hua et al., 2005), as well as in Xenopus at stages 45/46 (O'Rourke et al., 1994), though these studies did not examine branching in relation to target-directed motion. This dynamic but balanced branching behavior is consistent with navigation by selective branching, but not growth cone turning. Growth cone velocity remains non-zero after arrival at the termination zone (Fig. 4), while the growth cone targeting gradient approaches zero (Fig. 6), and the branch ratio becomes negative (Fig. 8). This persistent, but non-directed, growth cone motion may contribute to arbor extension, refinement and remodeling after arrival at the termination zone, past 3 to 4 dpf.

Previously, it was proposed that fish and frogs use "direct guidance" to their target zone in the tectum, while birds and mammals use an overshoot of the primary axon, followed by biased interstitial branching (reviewed in McLaughlin and O'Leary, 2005). Our observations lead us to conclude that zebrafish RGC axons are not guided directly towards a target zone by growth cone turning, but rather by selective branching. This is shown schematically in Fig. 10.

The time resolution of our study (10 minutes between frames) makes it difficult to track...
individual branches across frames. We cannot therefore determine whether the bias in branching towards the target is due to a bias in the direction of branch initiation, or a bias in branch stabilization following random initiation. This remains a topic for future study using timelapse imaging at higher temporal resolution.

**Comparison with previous zebrafish timelapse imaging studies**

A significant amount of previous work has examined targeting mechanisms en route to the tectum in zebrafish, but not in the tectum itself, prior to arborization. RGC axons cross the midline between 1.5 to 2 dpf. In the process of guidance towards the tectum, the roles of pioneer axons and axon-axon interactions (Pittman et al., 2008) and guidance cues (Fricke et al., 2001; Sakai and Halloran, 2006), have been assayed at 5 dpf. Timelapse and still images at 1.5 to 3 dpf (Hutson and Chien, 2002) have also been used to study the guidance of axons from the eye to the tectum. Branching is not usually seen in this stage of pathfinding and axons are dependent on guidance by growth cones (Hutson and Chien, 2002).

In the zebrafish tectum, a significant body of work has focused on the regulation and mechanisms of arborization dynamics. After axons arrive at their target locations, the branches that form their arbors continue to remodel. Branch dynamics at this time change as a result of several factors. Synaptogenesis is crucial to arbor rearrangements. Synapses stabilize existing branches and support new branch extension during 3 to 10 dpf (Meyer and Smith, 2006). Factors that affect synaptogenesis, including guidance cues, such as Slit1a/Robo2 during 3 to 5 dpf (Campbell et al., 2007), and synaptic proteins, e.g. glutamate transporters, assayed at 7 dpf (Smear et al., 2007) also affect the patterns of arborization. In addition, activity-dependent competition has been observed to limit the growth of axon arbors when an RGC’s activity level is lower than neighboring cells (Hua et al., 2005). In particular, lower numbers of new branches were seen forming between 4 and 5 dpf in single silenced axons, but there was no effect observed at earlier stages, i.e. during pathfinding (Hua et al., 2005). In separate studies from 3 to 7 dpf, silencing a single axon was found to increase the area of the silent arbor by increasing branch length but not number of branches (Fredj et al., 2010). At 3 to 5 dpf, global loss of activity increases the length of branches and area of arbors, without altering the rates of branch addition or deletion (Schmidt et al., 2000). Finally, retrograde signals, such as arachidonic acid, were observed to increase axon branch stability in zebrafish between 3 to 5 dpf (Leu and Schmidt, 2008). While not focusing on targeting mechanisms, other investigators have noted unexpectedly high rates of branching at 3 to 4 dpf (Meyer and Smith, 2006), and certain "bottlebrush" branching patterns of immature arbors at 4 to 5 dpf (Schmidt et al., 2004), which are consistent with our own results. However, these studies did not attempt to link the
process of branch remodelling to navigation across the tectum.

Indeed, few timelapse studies have focused on the targeting of individual RGC axons on the tectum. The strongest evidence for directed growth in zebrafish development comes from the pioneering work of Kaethner and Stuermer (Stuermer, 1988a; Kaethner and Stuermer, 1992, 1994). Of this work, the most influential has been the timelapse imaging performed in Kaethner and Stuermer (1992), which concluded “Growth cones traveled unerringly into the direction of their retinotopic targets without branching en route. At their target and only there, the axons began to form terminal arborizations...”. The authors noted occasional navigational errors that were corrected by branching, but concluded these were rare events. This work is currently used to support textbook explanations, such as “in frog and fish embryos, retinal axons project to roughly the correct topographic site in the tectum, and then begin to branch” (Sanes et al., 2012). These conclusions contrast significantly with our own, which may be at least partly due to the improved imaging methodology used in our study and partly due to a different interpretation of the branching seen during imaging. By updating the techniques used we were able to image axons for longer without phototoxic effects use confocal stacks to capture branches at different depths, and perform a rigorous quantitative analysis.

Comparison with studies in *Xenopus*, goldfish and newt

Some comparable work on retinotectal axon guidance has been performed in amphibians as well as other fish species. In *Xenopus*, timelapse imaging has been used to examine the early targeting behavior of axons en route to the tectum (Harris et al., 1987; Chien et al., 1993). As axons moved from the optic tract into the tectal neuropil, growth cones were observed to slow and become more complex, before stopping at or just past the target zone, not overshooting as in mammals/avian systems (Harris et al., 1987; McLaughlin and O’Leary, 2005). After forward movement slowed, backbranching occurred, with new branches sprouting from the axon shaft behind the leading growth cone and forming the basis of the axon’s initial arbor (Harris et al., 1987). Further timelapse imaging revealed the highly dynamic nature of the branching involved in the shifting connections at the target zone (Witte et al., 1996). Time-lapse imaging has also illustrated the interaction between branching and activity on the tectum (O’Rourke et al., 1994; Rajan et al., 1999; Ruthazer et al., 2003) along with the modulation of activity and remodelling by neurotrophic factors (Cohen-Cory and Fraser, 1995; Cohen-Cory, 1999). Arbor remodeling over days contributes to *Xenopus* retinotectal topography (O’Rourke and Fraser, 1990). Based on his observations in *Xenopus*, Fujisawa (1987) suggested that selective branching could subserve the ‘shifting connections’ that allow the retinotectal map to expand with a growing tectum, and that it could also be used to refine targeting.
Previous work in fixed tissue has also hinted that selective branching may play a role in a number of fish and amphibian species during the process of regeneration. In goldfish, observations of regenerating retinotectal axons suggested selective branching may be involved in target-directed orientation, whereas normally developing axons grew directly to their targets, with or without course corrections (Stuermer, 1988b,c; Stuermer and Raymond, 1989). In goldfish, timelapse imaging has suggested that targeting errors are corrected by random movements of axonal processes, where a single, predominantly unbranched, axon will grow without an apparent directional bias, but will retract and change direction more often if it is in an ectopic location (Dawson and Meyer, 2001). In newt, Fujisawa et al. (1982) also concluded that selective branching may be used in targeting error correction in regenerating retinotectal axons, but not in normally developing axons.

While the involvement of branching in targeting has been suggested by qualitative observations in some of the above studies, this has not been followed up by quantitative timelapse studies. Quantification of these observations is important because branching may be underestimated in purely qualitative analysis. For example we have shown that, early on in axon targeting, relatively few branches exist at any one point in time, but that branching is still highly dynamic and selective. Terminal arborization is typically considered to be a phase of extensive, localized branching. Our results suggest that, at least initially during this phase, branch selectivity continues, and that this phase actually represents just a slight shift in the balance between branches added and retracted, so that the arbor grows, and in a targeted manner.

**Mechanisms of branch control during pathfinding**

The similarities seen between the dynamics of axon branching during pathfinding and in subsequent remodelling suggest that previous studies of arbor rearrangements may inform our understanding of branching. In *Xenopus*, Ruthazer et al. (2003) determined that branches were selectively eliminated from incorrect targets, rather than stabilizing correct branches or only initiating branches in the correct area. Synapses providing points to both initiate and stabilizing branches as seen by Meyer and Smith (2006) as early as 3 dpf. This time in development correlates with when visual responses can first be evoked (Easter and Nicola, 1996; Niell and Smith, 2005) yet the first axons reach the tectum closer to 2 dpf (Stuermer, 1988a; Burrill and Easter, 1994). During this time, axons from later born RGCs are still growing into the tectum while the first axons to reach the tectum are forming functional connections, leading to an overlap in observed behaviors. However, the use of tetrodotoxin, which blocks electrical activity, was not seen to affect the gross targeting of zebrafish RGC axons (Stuermer et al., 1990) suggesting that the initial pathfinding does not depend as much on correlated activity.
as later arbor refinement processes (reviewed in Ruthazer and Cline, 2004). This could mean that, while the dynamics of branching do not drastically change, the mechanisms controlling branching may transition during development to become more dependent on activity.

At the molecular level, a number of different molecules have recently been shown to influence axonal branching in general. In particular many extracellular cues previously implicated in axon growth and guidance, such as Slits, class III semaphorins, ephrins, Wnts, neurotrophic factors and extracellular matrix proteins, are now known to guide, promote or inhibit axon branches as well (reviewed in Bilimoria and Bonni, 2012). Intracellular signalling molecules involved in branching include kinases, transcription factors, RhoGTPases, ubiquitin ligases and cytoskeleton associated proteins (Bilimoria and Bonni, 2012). In birds and rodents the interaction between levels of eph receptor on the axon and ephrin levels in the tectum appears to play a crucial role in restricting branching to topographically appropriate regions (Roskies and O'Leary, 1994; Yates et al., 2001). Our data suggest that slightly different molecular mechanisms might apply in the zebrafish, since branching is more promiscuous and continually increases as the axon moves across the tectum. An obvious possibility is that branches serve to sample whether the region of tectum at the branch tip has a more appropriate ephrin level than the site of branch initiation; however this remains to be tested.

**Computational models of branching in retinotectal map formation**

Axonal branching has been included in some computational models to expand the tectal region sampled (e.g. Willshaw and von der Malsburg, 1979; Overton and Arbib, 1982; Simpson and Goodhill, 2011). Models focused more particularly on branching include Yates et al. (2004) and Godfrey et al. (2009), though these were primarily designed to reproduce patterns seen in chicks and rodents. Tsigankov and Koulakov (2009) proposed more fundamental roles for branching, and investigated more generally what branching rules might be optimal for axons versus dendrites. Dendrite branching in particular was considered by Niell (2006), who investigated a 'synaptotropic' mechanism of selective stabilisation of filopodia from dendritic branches. Gierer (1987) described a variety of possible branching rules based on whether the growth cone sensed it was growing up or down a tectal gradient. Elucidating the precise computational mechanisms underlying the biased branching observed in our data provides an interesting direction for future work.

**Mechanisms of initial map development**

The data we have presented suggest that biased branching should be included as a guidance mechanism of zebrafish RGC axons during navigation across the tectum. While ‘directed
growth’ towards the tectal target does occur, this happens through exploratory branching and maintenance of branches oriented towards the target. Growth cones on these branches extend towards the target area without turning. Any course corrections are made through another round of branching and subsequent target-directed branch stabilization and growth cone extension. Through this iterative process, the axons navigate to their target location. We did not observe any ‘directed growth’ without branching.

We therefore suggest that classifying retinotectal mapping mechanisms for different species as ‘directed growth’ for amphibians and fish, and ‘selective/biased branching’ for mammals and chicks (McLaughlin and O’Leary, 2005) may need modification. Selective branching must always be accompanied by some form of target directed motion, otherwise progress towards the target cannot occur. Hence species that utilize selective or biased branching must also end up displaying target directed motion. It may be that there are no examples of pure growth cone navigation on the tectum / SC in the development of these maps, and that all rely at least in part on selective branching.

Our results also help to clarify the concept of ‘directed growth’ itself, which has not previously been well characterized. We distinguished target-directed growth cone motion, from orienting mechanisms such as growth cone turning or selective branching. We then showed that (1) selective branching, and not turning, is used to orient zebrafish retinotectal axons and branches, and (2) directed motion of these properly oriented growth cones occurs. To our knowledge, there is little evidence for growth cone turning in RGC axon navigation on the tectum / SC. One consequence of this is that the curved trajectories seen in some fish / amphibians (e.g. Fujisawa, 1981; Stuermer and Raymond, 1989), which have been previously been suggested in theoretical work to result from growth cone turning due to guidance cues (Gierer, 1983, 1987), and / or competitive influences (Simpson and Goodhill, 2011), may instead be due to branching-based course corrections, or potentially tectal growth effects on initially straight trajectories.

Although we argue that selective branching is important in zebrafish retinotectal map formation, but growth cone turning is not, the two concepts may share mechanistic links. Growth cone turning involves filopodia on one side of a growth cone extending and growing at the expense of those on the other side, which is mechanistically similar to selective branching, but on a smaller scale. This distinction may be more one of scale than of fundamental mechanism.

We can further refine the concept of selective branching in retinotectal / retinocollicular map development. As noted by Hua and Smith (2004), branch selection could proceed in two ways: sequentially, with a phase of exuberant branching, followed by widespread pruning; or simultaneously, with branch formation and elimination proceeding in tandem. Our work in fish, the work of Ruthazer et al. (2003) in Xenopus, and the recent work of Dhande et al. (2011)
in mice, suggests that selective branching and elimination in retinotopic map formation occurs simultaneously across all species.

**Conclusion**

We performed in vivo timelapse imaging of 2 to 4 dpf zebrafish retinotectal axon growth and targeting, using the BGUG (Brn3c:GAL4;UAS:mGFP) transgene (Xiao et al., 2005), in which retinal ganglion cells are sparsely labeled with mGFP. This allowed us to visualize individual axons in vivo for periods of 10-21 hrs without the phototoxicity associated with lipophilic dyes such as Dil (Kaethner and Stuermer, 1992; Potter et al., 1996). We looked quantitatively at the pathfinding dynamics of initial map targeting during 2 to 4 dpf, rather than rearrangements later in development. We found that growth cones move with mostly straight trajectories without turning. Instead, axons branch abundantly, with more branches oriented towards their target zone than away. Only a small percentage of these branches are kept. Growth cones at the tips of branches oriented towards the eventual termination zone grow ‘directly’ towards their target. This argues for an unexpectedly central role for selective branching during pathfinding in zebrafish retinotectal axons. Our work clarifies the notion of directed growth of axons by quantifying growth cone motion, turning, and branching, and analyzing these quantities relative to the axons’ targets. These results suggest that, contrary to previous belief, selective branching during initial pathfinding may be a ubiquitous feature of guidance during retinotectal / retinocollicular map formation across a range of species, rather than being restricted to birds and mammals.

**Conflict of Interest Statement**

The authors certify that there are no conflicts of interest.

**Role of Authors**

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: HS, ES, GG. Acquisition of data: HS. Analysis and interpretation of data: HS, EK, ES, GG. Drafting of the manuscript: HS, EK, ES, GG. Critical revision of the manuscript for important intellectual content: HS, EK, ES, GG. Statistical Analysis: HS. Obtained funding: ES, GG. Study supervision: ES, GG.
References


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Table captions

Table 1: **Antibody Characterization.** Previously characterized anti-acetylated tubulin and GFP were used in this work.

Table 2: **Description of live imaging data.** Ten axons from six larvae provided videos with axons that met the criteria for inclusion.

Table 3: **Branch addition and retraction values.** For each axon (rows) total number of branches added, retracted, and net number of branches are shown (left side of table), as well as average rates of branch addition and loss (right side of table). Total branch numbers and averages were calculated over the entirety of the timeseries data for each axon.

Table 4: **Growth cone velocity data.** For individual axons (rows) maximum velocity recorded and average velocity over its trajectory are tabulated in $\mu m/hr$. The average velocity contains pooled data from multiple growth cones within each individual axon. Minimum velocity for all axons was zero, as all axons paused for at least one frames. Maximum velocity for all axons, and mean velocity and standard error over all axons is shown in the bottom row.
Figure captions

Figure 1: **Image sequence from an example timelapse movie.** Example movie frames from timelapse imaging of a zebrafish embryo containing BGUG and Atoh7:GAL4. RGC axons are labeled with mGFP (green) and there is notable skin autofluorescence. R, rostral; C, caudal; M, medial; L, lateral. Temporal progression is left to right, top to bottom. Sequence of images every 2.5hrs of a 20hr movie, corresponding to the period of 56-76hpf. Arrows indicate axons traced in this movie. In the first panel (t = 0hrs) the ipsilateral eye is marked with a dashed white line, and optic tectum (OT) is outlined with a dotted white line. The first labeled axon to arrive on the tectum (purple arrow), was followed shortly by a second (orange arrow), and both contributed to the timelapse data. Other axons entered the tectum at later stages (white arrowheads), but were not analyzed. Scale bar 50µm.

Figure 2: **Image analysis and axon tracing for in vivo timelapse and fixed immunostained imaging.** (A-D) A single frame of data and analysis from one of the timelapse movies. (A) Raw image. Three axons were already on the tectum (arrows), and two were entering the tectum (arrowheads). The axon enclosed by the blue box is considered in the rest of the figure. Scale bar 50µm. R, rostral; C, caudal; M, medial; L, lateral. (B) Cropped image, zoomed in on the selected axon in A. (C) The tracing of the axon in A & B, with convex hull fit (red) to the branch end points (black ‘x’s). Free branch endings were used in the analysis of total arbor area and centroid location. (D) Distribution of branch lengths in this frame including two branches that do not have free endings. (E,F) Zebrafish embryo at 5dpf; fixed, permeabilized, and immunostained using anti-acetylated tubulin (magenta) and anti-GFP (green). (E) 10x magnification. Eyes, forebrain (Fb), midbrain (Mb), and cerebellum (Cb) / hindbrain (Hb) are visible. Labeled RGC arbors were visible in both tecta. Arbor of interest marked with white arrow, others marked by white arrowheads. Scale bar 100µm. (F) 20x magnification. Two RGCs were visible on the neuropil of the optic tectum. The neuropil had a scalloped, meshwork appearance, and was surrounded by radial projections. Axons entered the tectum in one of several fascicles (F, white bracket) rostral to the tectum. Arbor of interest marked with white arrow, another axon is marked by a white arrowhead. Scale bar 50µm.

Figure 3: **Highly dynamic branch addition and subtraction are tightly balanced.** Branching dynamics for a single example axon (top row, A-D) and averaged over all axons (bottom row, E-H). Vertical dashed line in E represents the time of target arrival, t=0, about which data were aligned. (A,E) Total number of branches increased with time for an individual axon (A) and for pooled axon data (E). (B) Number of branches added (positive values) or retracted (negative values) from the previous frame. Branch addition and loss were tightly coupled, yet the small positive bias resulted in the net branch
addition in A. (C,D,F) A large number of branches were added and retracted in individual axons (C), but the net number of branches was comparatively small in individual axons (D) and over all axons (F). (G) The average number of branch additions (black) and losses (grey) increased steadily with time. We did not observe any sudden change in dynamics when the axon arrived at the termination zone and began to arborize. (H) Rates of addition (black) and rates of loss (grey) were tightly balanced within axons, but with addition slightly greater than retraction, giving the positive net differences in branch numbers (C,D,F). Rates calculated using total branch numbers ('x') were smaller than those calculated by branch order ('o'), as the latter method can better distinguish between individual branches on the basis of their order (see Methods). Rates normalized to arbor length (H), were similar across axons. Error bars in E, F and G represent SEM.

Figure 4: Growth cone velocity is consistent throughout navigation and among axons. Trajectories and velocities from a single example axon (A-C), and data from all axons (D-F). (A) Growth cone trajectories across the tectum. Different colors correspond to different growth cones. Initially only one growth cone was present (blue), which was then replaced by two short-lived growth cones (red, green), followed by two more growth cones (purple, cyan). (B) Instantaneous velocity varied among growth cones, and no trends were obvious for individual trajectories. Colors correspond to growth cones in (A). (C,F) Averaged instantaneous velocity of growth cones from the same axon as in A & B showed no obvious trends with time (C), and this was also true of the pooled axon / growth cone data (F; error bars are SEM, t=0 is the time of target arrival, about which data were aligned). (D,E) Mean velocities for individual axons (D) and growth cones (E) were similar among axons and growth cones. Error bars are SEM.

Figure 5: Growth cone trajectories do not show significant turning towards their targets. (A,B) Trajectories of all growth cones plotted with a common starting point at the origin (A, growth is radially outward), and plotted with a common termination zone at the origin (B, growth is radially inward). Different colors indicate different growth cones. Most trajectories were straight, with little to no obvious turning towards their target. (C-E) Growth cone turning angles. Positive values indicate turning towards a growth cone’s target, negative values indicate turning away from the target. (C,D) Timeseries of instantaneous growth cone turning angles relative to their targets were centered around zero, in individual axons (C, different colors are different growth cones), and averaged over all axons (D, error bars are SEM). (E) The cumulative distribution of average turning angle for growth cones is centered around zero. Error bars are SEM. (F) Turning ratio for growth cone trajectories. A positive ratio indicates a growth cone made more turns towards its target than away, and a negative ratio indicates a growth cone made more turns away from its target than towards. The cumulative distribution of turning
ratios is also centered around zero.

**Figure 6: Growth cones exhibit target-directed motion.** Axon and growth cone motion relative to their targets is shown for a single axon (A-C) and for all axons (D-F). (A,B) The axon arbor centroid (A) and most growth cones from the same axon (B, different growth cones shown in different colors) move steadily towards their target. One growth cone (green) moved parallel to the target, while after arrival at the target (t=0) two growth cones moved away from the target. Target-directed growth cone motion was quantified by the targeting gradient, TG, which is the pooled and averaged slopes of the curves shown in B (i.e., the gradient of the target approach curve). A negative gradient indicates motion towards the target, and a positive gradient indicates motion away from the target. The targeting gradient remained negative for most of the time imaged, until target arrival. (D) The time-averaged targeting gradients were significantly negative for 8/10 axons studied (cumulative distribution shown; error bars are SEM). Axon in A-C shown with red dot. (E,F) Similarly to the axon in B & C, the pooled axon data show that the majority of growth cones had negative slopes (E), and that the targeting gradient was predominantly negative during navigation to the target (F). Vertical dashed lines in E & F represent the point t=0 about which movies were aligned.

**Figure 7: Selective branching guides axons towards their target area** Individual tracings for 4 axons (A - D) showing selective branching in retinotectal axon guidance. The axons initially grew straight, then paused and extended branches in both directions, then sent out branches with an improved orientation with respect to its target at approximately 90° to the primary shaft, and then sent out higher order branches directly to the termination zone (light gray ‘x’). (A) Times elapsed between frames: 120, 130 and 110 minutes. (B) Time elapsed between frames: 50, 50, and 150 minutes. (C) Time elapsed between frames: 70, 90, and 100 minutes. (D) Time elapsed between frames: 180, 120, and 200 minutes.

**Figure 8: Axons preferentially stabilize branches oriented towards their targets.** The branch ratio timeseries for the axon in Fig. 7D remained positive for the majority of the time it was imaged, until arrival at its target (triangles and crosses indicate time and branch ratio of four panels in Fig. 7D ). Dashed line indicates a 2hr gap where two timelapse movies were joined. Branch ratio can vary from -1 to +1, with positive values indicating more branches are oriented towards its target than away. (B) The cumulative distribution of time-averaged branch ratios shows that 7/10 axons had a significantly positive ratio, and 1/10 had a significantly negative branch ratio (error bars are SEM; axon in Fig. 7D and Fig. 8A is shown with a cross). (C) Pooled branch ratio timeseries show that the positive trend seen in A also held across all axons imaged (black line is the mean, grey error bars are SEM). (D)
Selective branching (measured by mean branch ratio, grey) and directed growth (measured by mean target approach gradient, black) were negatively correlated. Vertical dashed lines in C & D represent the time of target arrival, t=0, about which data were aligned.

Figure 9: **Variation of dynamic variables and guidance measures by tectal position.** Variation by rostrocaudal (A, C, E), mediolateral (B, D, F) position; by ∆ML (the difference between mediolateral point of entry, and mediolateral stable position, G); and by initial distance to termination zone (H) (distance from point of tectal entry to termination). A rostrocaudal (RC) position of zero is at the rostral tectal border, and one is at the caudal border. A mediolateral (ML) position of zero is at the medial tectal border, and one is at the lateral tectal border. ∆ML is in units of normalized mediolateral tectal extent. Rostrocaudal position, mediolateral position, and ∆ML are determined from fixed animals at 4.5-6.5dpf. Distance to termination zone was derived from timelapse imaging. Individual data points are plotted with linear fits, lines with significant correlations are thicker. A & B: Average velocities; no significant trends were seen. C & D: Rates of extension and retraction. Significant trends were seen for both extension ($r = -0.64$, $p = 0.047$) and retraction ($r = -0.81$, $p = 0.004$) vs rostrocaudal position (C), but not vs mediolateral position (D). (E-H) Variation in guidance measures: branch ratio and targeting gradient. E: There was a non-significant association between rostrocaudal position and both guidance measures. F: No association between mediolateral position and guidance measures was evident. G: There was a non-significant association between ∆ML and both guidance measures. H: There was a strong and significant correlation between initial distance to target, and both measures of guidance: branch ratio: $r = 0.83$, $p = 0.003$; targeting gradient: $r = 0.77$, $p = 0.01$.

Figure 10. **Models of guidance across the tectum.** Different species use different variations of pathfinding mechanisms to establish the initial retinotopic map on the tectum / superior colliculus. **Top row:** In chicks / mammals, axons grow caudally across the superior colliculus and overshoot the target area (dashed ovals). Interstitial branches then extend, and once a branch reaches the termination zone, further branching and arborization begins. Incorrectly oriented branches and the axon overshoot are pruned back. **Middle row:** Previously, fish / frogs were thought to depend on “direct guidance” by the growth cone, which would guide an unbranched axon to the target zone. Subsequently, the axon would initiate branching to form an arbor. **Bottom row:** We suggest a new model for fish that takes into account the biased branching seen during pathfinding. As the axon extends into the tectum, dynamic branches are added and eliminated, with a bias for branches to project towards the target zone. Branches have a high turnover rate and most are lost. Branches continue to be added once the axon halts forward progress and starts to arborize in its termination zone. Thus, both mammals/chicks and fish rely on selective branching, and although the patterns of growth are different, the mechanisms of pathfinding may be more similar than previously believed. In the summary figures for each condition (right column) green lines represent branches that have been maintained, and red the portions of the
axon that have been eliminated over time. Axons grow in from the rostral edge of the tectum / superior colliculus (top). Schematics for development of chick/mammal retinotectal growth and a classic view of fish / frog retinotectal arborization were adapted from McLaughlin and O’Leary (2005).
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Table 1: Antibody Characterization
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Table 2: Description of live imaging data.
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Table 3: Branch addition and retraction values.
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Table 4: Growth cone velocity data.
Figure 1: Image sequence from an example timelapse movie.
Figure 2: Image analysis and axon tracing for in vivo timelapse and fixed immunostained imaging.
Figure 3: Highly dynamic branch extension and retraction are tightly balanced.
Figure 4: Growth cone velocity is consistent throughout navigation and among axons.
Figure 5: Growth cone trajectories do not show significant turning towards their targets.
Figure 6: Growth cones exhibit target-directed motion.
Figure 7: Selective branching guides axons towards their target area.
Figure 8: Axons preferentially stabilize branches oriented towards their targets.
Figure 9: Variation of dynamic variables and guidance measures by tectal position.
Figure 10: Models of guidance across the tectum.