

Supporting Information

Hiramoto and Cline 10.1073/pnas.1416953111

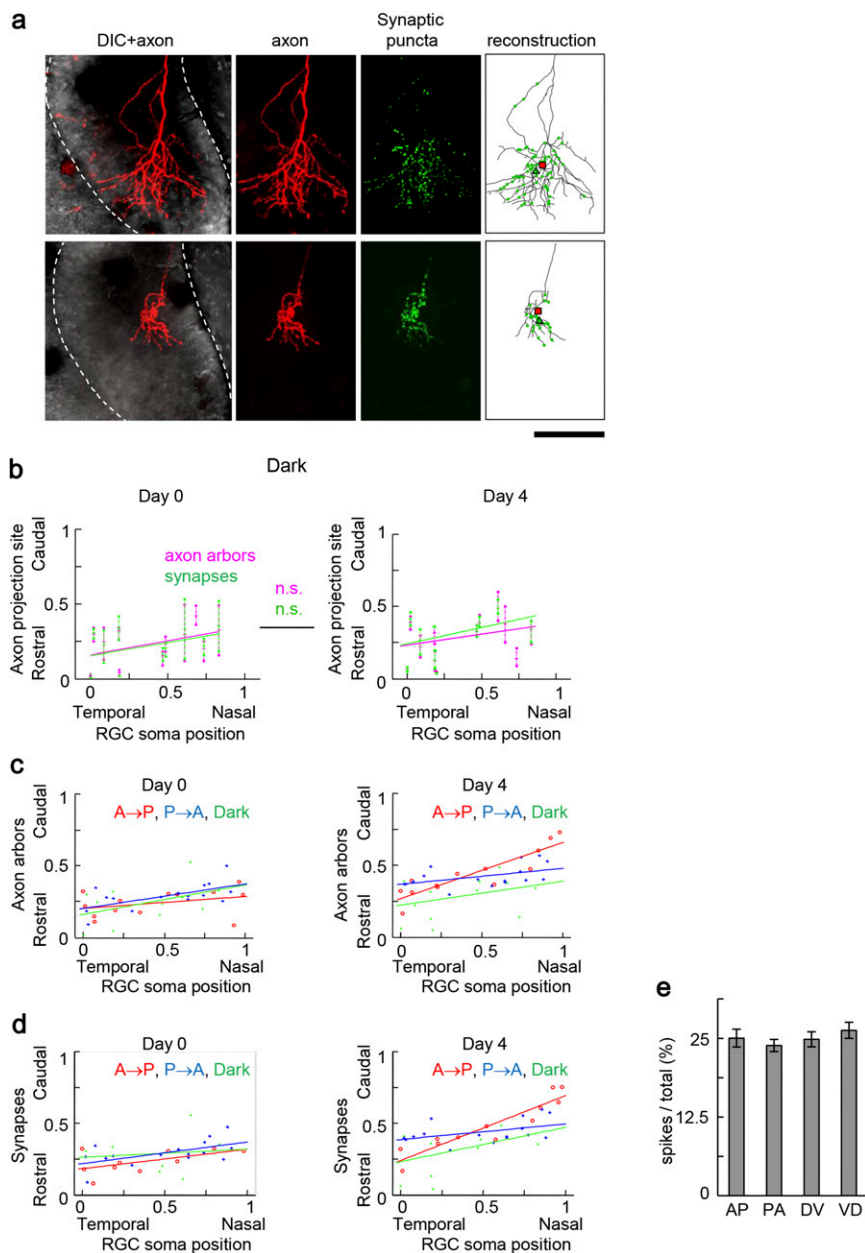


Fig. S1. Retinotopic maps fail to refine in the dark, pertaining to data in Fig. 1. RGCs were labeled with TdTomato and Synaptophysin-GFP by focal electroporation in stage 41 tadpoles, and animals were maintained in the dark until imaging experiments began 4 d later. Animals are taken out of the dark briefly to image the axons. Images were collected of single retinotectal axons and presynaptic puncta at day 0 and 4 d later. Positions of labeled RGC somata and retinotectal axon arbors are determined relative to the temporal-nasal axis of the retina and the rostrocaudal axis of the tectum, respectively. (A) Examples of two axon arbors after dark-rearing. The *Left* panels show images of axons superimposed on a DIC image of the tectum, shown outlined. The *Middle* panels show images of the axon and Synaptophysin-GFP puncta. The *Right* panels show reconstructions of the axons (black lines) with presynaptic puncta (green). Centers of mass for the axons (red square) and synapses (blue triangles) are superimposed on the axon drawing. The labeled RGC somata were located at the 2nd and 61st percentiles along the temporal-nasal axis of the retina. (Scale bar, 100 μm .) (B) Retinotopic maps at day 0 and day 4. Relative RGC soma position along the nasotemporal axis in the retina was plotted against the relative positions of the center of mass of the axons (magenta) and synaptic puncta (green) along the rostrocaudal axis in the tectum. Bars indicate 25th percentiles. The soma positions and axon projection sites are not significantly correlated on either day 0 (axon arbors, $P = 0.16$, $R = 0.46$; synapse, $P = 0.23$, $R = 0.42$) or day 4 (axon arbors, $P = 0.16$, $R = 0.41$; synapse, $P = 0.1$, $R = 0.51$; $n = 11$). Retinotopy was not significantly different between day 0 and day 4 [ANCOVA, $P > 0.9$ (axons) and $P > 0.7$ (synapses)]. It is interesting to note that the variance of the axon arbor positions after dark-rearing was significantly larger than after P→A motion stimulus ($P = 0.03$). (C and D) Overlays of centers of mass and regression lines of

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retinotopic maps of axon arbors and synapses from animals exposed to A→P and P→A motion stimuli or reared in the dark. Data are replotted from Fig. 1 D and E and Fig. S1B. The topographic maps at day 0 are similar for the three conditions, but the axons from the nasal RGCs in animals that received A→P visual experience moved 48 μm further into caudal tectal territory than nasal RGCs in animals with P→A visual experience. On the other hand, the arbors from temporal RGCs in animals with P→A visual experience moved 18 μm toward caudal tectum compared with temporal axons with A→P motion stimulus, which occupied relatively rostral tectal locations. The overall rostrocaudal length of the tectal neuropil is about 180 μm . (E) Relative number of spikes evoked by moving bar stimuli recorded from the optic tract. A moving bar drifting from A→P, P→A, D→V, and V→D was presented to the eyes. For each direction, the numbers of spikes for 10 trials were averaged. Data from 10 animals were averaged. The numbers of spikes in response to each directional stimulus were divided by the total spikes in response to all directions. The following methods were used: A 4.5 cm \times 4.5 cm back projection display was presented 25 mm from the animals' eyes. A black bar with 1.67-cm thickness moving at 130 mm/s was displayed on the screen. The intensity of the display was 0.045 mW/cm².

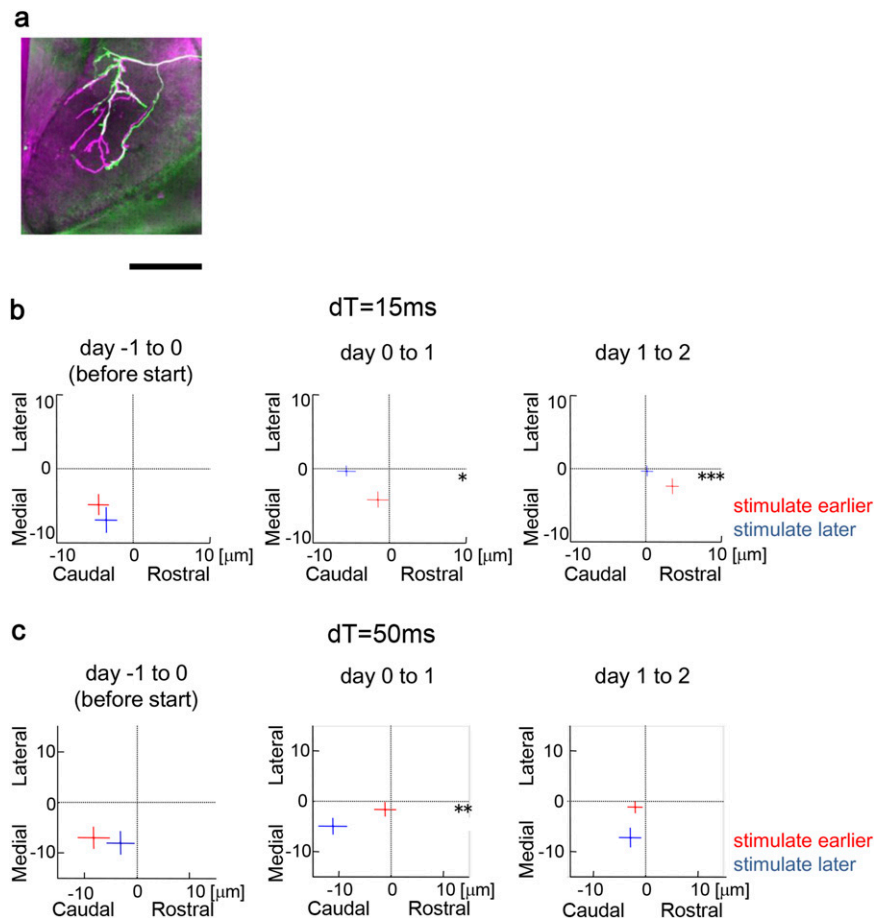


Fig. S2. Pertaining to data in Fig. 3. Changes in branch tip positions for each day over 2 d of visual experience. (A) Overlay of aligned images of the sample shown in Fig. 3D at day 0 (green) and day 1 (purple). (Scale bar, 100 μm .) Converging retinotectal axons were activated sequentially by presenting a visual stimulus to one eye or the other with interstimulus intervals of 15 or 50 ms, as shown in Fig. 3. (B and C) Plots of the mean of the shift in branch tip positions and the SE of mean, indicated as the length and height of the bars in the cross symbols. Red and blue crosses correspond to data collected when the eye with the labeled axon was stimulated earlier (red) or later (blue) than convergent inputs from the other eye. Before starting the visual stimulation protocol, when animals are kept in the dark (B and C, day 1–0), branches of all axon groups shift to a comparable extent toward the caudomedial tectum ($dT = 15$ ms and 50 ms; $P > 0.1$). This likely reflects continued extension of axons after entering the tectum. (B) During the first and second days of visual experience with the 15-ms interstimulus interval protocol, the shifts in branch tip positions for earlier- and later-stimulated axons were significantly different (day 0–1, $P < 0.01$; day 1–2, $P < 0.001$), however the direction and magnitude of branch shifts differed between the 2 d of visual experience. During the first day of visual experience (day 0–1), branches in the later-stimulated axons (blue) shifted toward the caudal tectum, and on average did not change their positions during the second day of visual experience (day 1–2). By contrast, branches in the earlier-stimulated axons (red) on average shifted toward the medial tectum during the first day of stimulation (day 0–1) and toward rostral tectum on the second day of stimulation (day 1–2) as seen in the example shown in Fig. 3C. When the interstimuli interval was 50 ms, we only detect a significant difference in the shifts of branch tip positions between day 0–1, when the branches in the later-stimulated axons (blue) shifted toward the caudal tectum, whereas on average the branch positions in the earlier-stimulated axons (red) were stable (C, day 0–1). During the next day (C, day 1–2), branches in the later-stimulated axons on average shifted less toward caudal tectum. Mean \pm SEM for x and y = $[-4.5, -4.9] \pm [1.4, 1.4]$ (day 1–0, before 15 ms earlier), $[-3.5, -6.9] \pm [1.5, 1.7]$ (day 1–0, before 15 ms later), $[-1.5, -4.2] \pm [1.4, 1.1]$ (day 0–1, 15 ms earlier), $[-5.6, -0.33] \pm [1.2, 0.70]$ (day 0–1, 15 ms later), $[3.5, -2.4] \pm [0.87, 1.0]$ (day 1–2, 15 ms earlier), $[0.18, -0.33] \pm [0.48, 0.72]$ (day 1–2, 15 ms later), $[-8.2, -7.0] \pm [3.1, 2.2]$ (day 1–0, before 50 ms earlier), $[-3.1, -8.1] \pm [2.7, 2.3]$ (day 1–0, before 50 ms later), $[-0.89, -1.3] \pm [1.7, 1.1]$ (day 0–1, 50 ms earlier), $[-9.0, -4.0] \pm [2.2, 1.4]$ (day 0–1, 50 ms later), $[-2.0, -1.2] \pm [1.5, 1.2]$ (day 1–2, 50 ms earlier), and $[-3.0, -7.2] \pm [2.2, 2.0]$ (day 1–2, 50 ms later). Data were collected from five to eight arbors per condition, and arbors had 78–182 branches. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

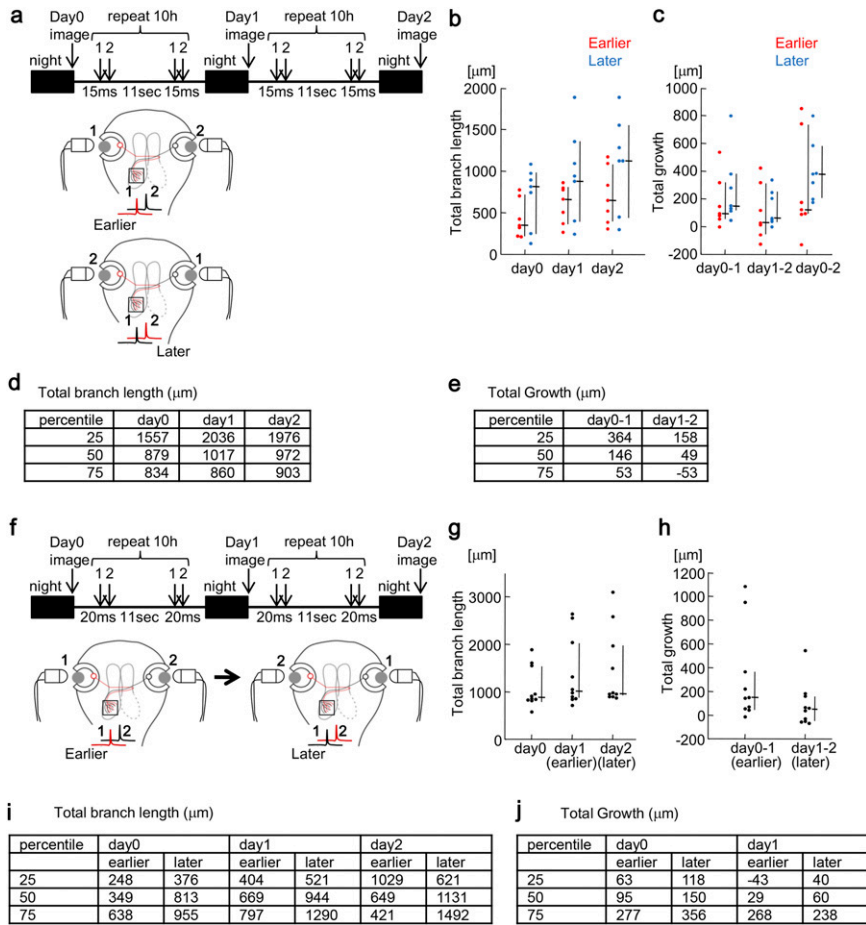


Fig. S3. Pertaining to data in Figs. 3 and 4; morphometric analysis of axon branch length and growth rate in binocular tecta. (A–E) The 15-ms interstimulus interval protocol was applied for 1 or 2 d. The eyes with labeled axons were stimulated earlier or later than the other eye. There was no significant difference between earlier- and later-stimulated axons in either total axon branch length (B, Wilcoxon, $P[\text{day 0 day 1 day 2}] = [1\ 1\ 1]$; $n = 7$) or growth rate (C, Wilcoxon, $P[\text{day 0-1 day 0-1 day 0-2}] = [0.38\ 0.53\ 0.21]$; $n = 7$). (D and E) Median, 25 and 75 percentile values from B and C, respectively. (F–J) Stimuli with a 20-ms interstimuli interval were applied. The eyes with labeled axons were stimulated earlier on the first day (earlier), then stimulated later on the second day (later). There was no significant difference between earlier- and later-stimulated axons in either total axon branch length (G, Wilcoxon, $P[\text{day 0 vs. day 1, day 1 vs. day 2}] = [0.27\ 0.68]$; $n = 10$) or growth rate (H, Wilcoxon, $P[\text{day 0-1 day 1-2}] = [0.09\ 0.09]$; $n = 10$). (I and J) Median, 25 and 75 percentile values from G and H, respectively.

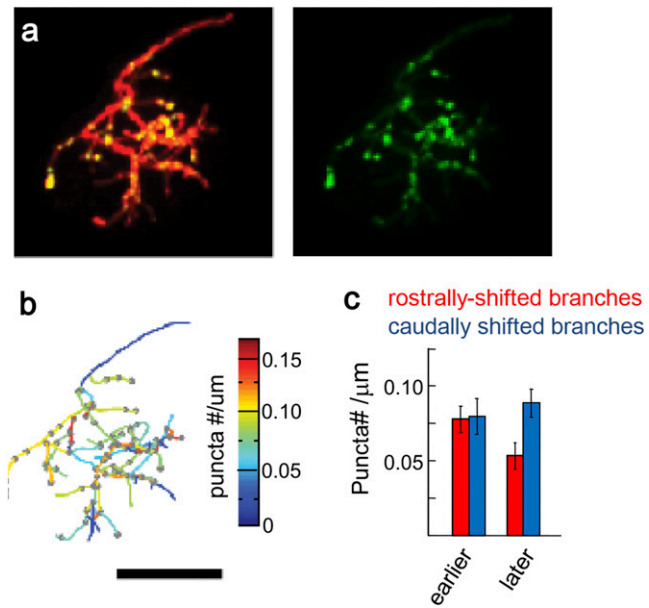


Fig. S4. Pertaining to data in Fig. 3; dynamic branches form synapses. (A) In vivo image of a retinotectal axon expressing cytosolic Td-Tomato and Synaptophysin-GFP to label presynaptic puncta. (B) Branches are color-coded according to their puncta densities, shown in the scale (Right). (C) Comparison of the puncta density in branches that shifted toward the rostral or caudal regions of the arbor over 2 d of visual stimulation. Branches in earlier-stimulated arbors that shifted toward more rostral or caudal positions formed synapses with comparable density. Synapse density in branches that shifted rostrally in later-stimulated arbors was significantly lower than the synapse density in branches in the same arbors that shifted caudally [$P < 0.01$, $n = 53$ branches (rostral) and 83 branches (caudal) in seven axons].

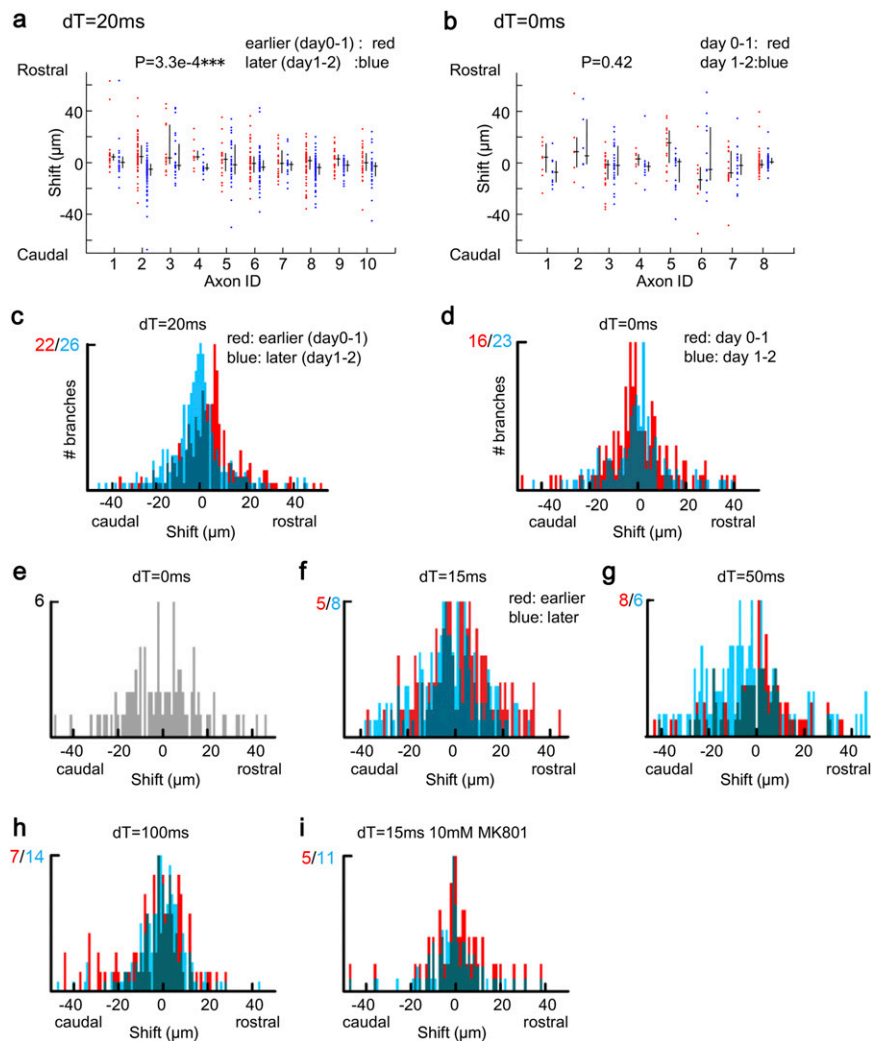


Fig. S5. Pertaining to data in Figs. 3 and 4; analysis of branch movements. (A and B) Scatter plots showing the magnitude of each branch shift for each axon stimulated with dT = 20 ms or dT = 0 ms interstimulus intervals. Median \pm 25 percentiles are shown to the right of individual data points for each condition. (A) Eyes with labeled RGCs were stimulated 20 ms earlier (red, day 0–1) or later (blue, day 1–2) than the other eye (Fig. 4). (B) Both eyes were stimulated simultaneously (red, day 0–1; blue, day 1–2). In the dT = 20 ms condition, the median shift in branch tip positions of axons was significantly different between the earlier and later stimulation periods ($P = 3.3 \times 10^{-4}$, $n = 10$). The shift between day 0–1 and day 1–2 was not significantly different in the dT = 0 ms condition ($P = 0.42$, $n = 8$). (C–I) Histograms of the direction and magnitude of shifts of individual branch tips in all axons for different stimulus conditions. (C and D) Branch shifts for data from dT = 20 ms and 0 ms, from the experiment shown in Fig. 4 and Fig. S5 A and B. (E–I) Branch shifts for data from experiments shown in Fig. 3 for dT = 0 ms, 15 ms, 50 ms, 100 ms, and 15 ms with MK801 for 2 d. Red and blue correspond to the data from the eye stimulated earlier (red) or later (blue) than the other eye.

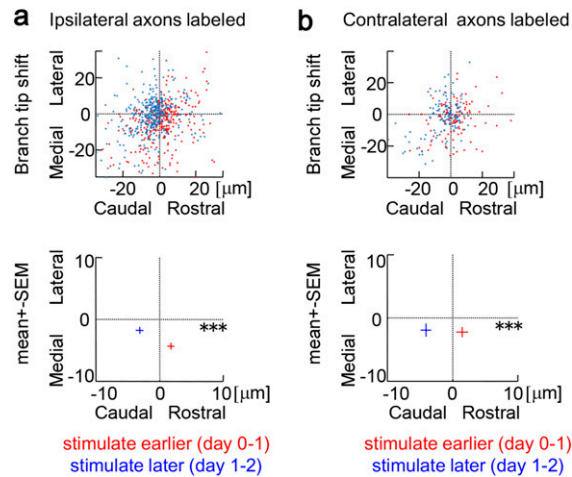


Fig. S6. Pertaining to data in Fig. 4; the sequential order of eye stimulation governs axon arbor branch rearrangements in axons originating from eyes ipsilateral or contralateral to the binocular tectum. (A and B) Plots (Top) of the shifts in branch tip positions in axons from animals with binocular tecta in which the eyes were stimulated sequentially with $\Delta T = 20$ ms. (A) Data from axons originating from the eye ipsilateral to the binocular tectum. (B) Data from axons originating from the eye contralateral to the binocular tectum. Red data points show the shift in branch tip positions when the labeled axon was stimulated earlier than convergent inputs from the other eye, and blue data points show the shift in branch tip positions when the labeled axon was stimulated later than inputs from the other eye. Summary plots (Bottom) show the mean and SEM of the shift in branch tip position (see Table S3 for values). SEM is shown as the length of the bars in the symbols. When the eyes were stimulated with $\Delta T = 20$ ms, branch tip positions shifted significantly for both ipsilateral axons ($P < 0.001$, paired *t* test, $n = 440$ from 10 axons) and contralateral axons ($P < 0.001$, paired *t* test, $n = 172$ from six axons).

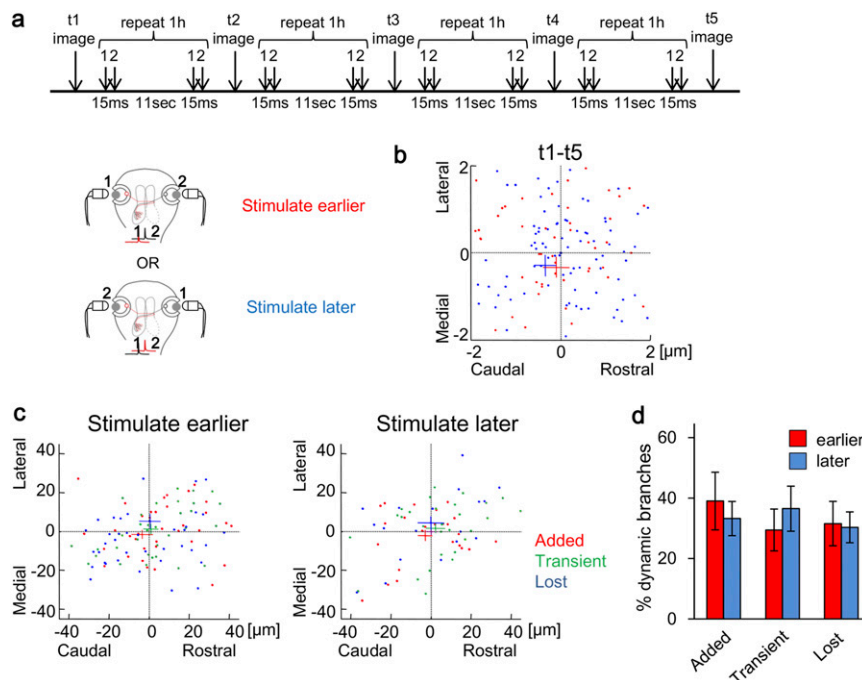


Fig. S7. Pertaining to data in Figs. 3 and 4; dynamic shift in axon arbor branch positions is not detected after 4 h of sequential eye stimulation. (A) Schematic of experimental protocol. Axons in binocular tecta were imaged once an hour over 4 h while animals received visual stimulation provided sequentially to the left and right eyes with interstimulus intervals of 15 ms at 0.09 Hz for 4 h. (B) Plot comparing the shifts in branch tip positions in axons from animals stimulated with 15 ms earlier (red) and 15 ms later (blue). The red and blue crosses show the mean and SEM of the populations of branch tips. No significant difference in the change in branch tip positions can be detected when data are analyzed over 4 h [$P > 0.2$, $n = 239$ branches in eight axons (15 ms earlier) and $n = 271$ branches in six axons (15 ms later)]. (C) The shifts in dynamic branches in axons stimulated 15 ms earlier or later than convergent inputs from the other eye. The plots show the positions of the stabilized, transient, and lost branches relative to the center of mass of all the new branches. No significant differences in the relative distributions of transient and stable branches are detected. $P > 0.2$ for stabilized, transient, and lost branches for earlier- and later-stimulated axons. Earlier-stimulated axons, $n = 128$ branches in eight axons; later-stimulated axons, $n = 77$ branches in six axons. (D) Percentage of dynamic branches in each category for earlier- and later-stimulated axons. Stabilized, 39 ± 10 (earlier) and 33 ± 6 (later); transient, 29 ± 7 (earlier) and 36 ± 7 (later); eliminated, 32 ± 7 (earlier) and 30 ± 5 (later).

Table S1. Mean and SEM, for data in Fig. 3 F-I'

dT	Condition	Mean shift \pm SEM, μm , x, y	n, branches, axons
0 ms		0.45 \pm 2.1, -3.6 \pm 2.3	167, 8
15 ms	Earlier	2.1 \pm 1.7, -6.6 \pm 1.4	144, 7
	Later	-5.4 \pm 1.5, -0.67 \pm 0.91	219, 7
50 ms	Earlier	-3.0 \pm 2.2, -2.7 \pm 1.6	110, 8
	Later	-11 \pm 2.3, -9.9 \pm 1.9	134, 5
100 ms	Earlier	-3.2 \pm 1.2, -4.0 \pm 1.5	145, 8
	Later	-1.7 \pm 0.67, -3.9 \pm 0.82	284, 5

Table S2. Wald-Wolfowitz runs test, for data in Fig. 4

Axon ID	Earlier, day 0-1	Branch number	Later, day 1-2	Branch number
dT = 20 ms				
1	-0.78	18	-0.32	18
2	-0.20	56	-0.051	75
3	-0.56	14	-0.087	23
4	-1.17	10	-0.92	11
5	-0.40	23	-0.065	27
6	-0.12	46	-0.22	56
7	-0.26	19	-0.41	16
8	-0.13	62	-0.032	84
9	-0.15	29	-0.11	27
10	-0.34	25	-0.26	30
dT = 0 ms				
1	-0.82	8	-0.92	9
2	-2.9	6	-0.92	6
3	-0.41	26	-0.22	25
4	-0.92	7	-0.18	11
5	-0.78	15	-0.32	16
6	-0.82	8	-0.82	11
7	-0.41	17	-0.41	17
8	-0.065	46	-0.20	45

We tested whether the direction of branch tip movements are independent using the Wald-Wolfowitz runs test. Tables of the z values calculated by the Wald-Wolfowitz runs test are shown. The data are not random when the absolute value of z is larger than 1.96 [$=Z(1-\alpha/2)$; $\alpha = 0.05$]. The branch movement data were arranged in a series defined by proximity between branch tips before stimulation (the branch #N+1 is the branch whose tip is closest to the branch #N). The first branch in the series was chosen so that the variance of the intertip distances was the smallest. To apply the Wald-Wolfowitz runs test, directionality of the shift along the rostrocaudal axis was analyzed. The data show that the branch tip movements were random for all axons with more than six branches.

Table S3. Mean and SEM, for data in Fig. 4 D'-G' and Fig. S6

dT	Condition	Mean shift \pm SEM, μm , x, y	P, t test; n, branches, axons
0 ms	Day 0-1	0.78 \pm 1.9, -1.4 \pm 2.2	0.69, 0.80; 167, 8
	Day 1-2	-0.32 \pm 1.5, -2.4 \pm 2.2	
20 ms (all)	Day 0-1 (earlier)	1.7 \pm 0.48, 3.8 \pm 0.47	7.0e-14, 6.7e-3; 612, 16
	Day 1-2 (later)	-3.4 \pm 0.48, 1.8 \pm 0.51	
20 ms (nasal)	Day 0-1 (earlier)	2.3 \pm 0.60, 3.2 \pm 0.56	9.1e-11, 0.15; 405, 11
	Day 1-2 (later)	-3.4 \pm 0.63, 1.8 \pm 0.67	
20 ms (temporal)	Day 0-1 (earlier)	0.57 \pm 0.77, 4.9 \pm 0.86	2.3e-4, 3.7e-3; 207, 5
	Day 1-2 (later)	-3.0 \pm 0.79, 1.8 \pm 0.71	
20 ms (ipsilateral)	Day 0-1 (earlier)	2.5 \pm 0.62, -5.9 \pm 0.62	9.1e-10, 3.1e-3; 440, 10
	Day 1-2 (later)	-3.5 \pm 0.61, -2.0 \pm 0.58	
20 ms (contralateral)	Day 0-1 (earlier)	1.5 \pm 0.92, 2.2 \pm 0.81	1.4e-5, 0.8; 172, 6
	Day 1-2 (later)	-4.1 \pm 0.81, 1.9 \pm 1.1	