Figures and figure supplements

Filopodial dynamics and growth cone stabilization in *Drosophila* visual circuit development

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Figure 1. Development of Drosophila pupal brains in an imaging chamber. (a) Timeline of photoreceptor circuit formation during brain development and the periods accessible by live imaging. (b) Ex vivo imaging chamber, top (left) and side (right) views (see Figure 1—figure supplement 1 for step-by-step assembly). (c-h) Changes in brain morphology during development ex vivo v. in vivo. (c,f) Pupal brains dissected at P + 24% and P + 50%. (d, g) The same brains after 24 hr of development ex vivo. (e, h) Brains that were dissected from pupae collected at P + 24% and P + 50% and aged in parallel to the ex vivo brains. See Figure 1—figure supplement 2 for comparison with free-floating cultures. (i-p) Optic lobe development ex vivo v. in vivo (i'-p') magnified details of (i-p). All photoreceptors express CD4-tdGFP. Initial layer separation (P + 24% + 19 hr) occurs ex vivo (i', j') similarly to the in vivo controls (k', l') aged in parallel (blue arrows: R8, green arrows: R7). Lamina rotation (red arrows) observed in vivo (k, l) is defective ex vivo (i, j). Final layer formation and lamina expansion (P + 40% + 18 hr) occurs similarly ex vivo and in vivo, (m'-n') v. (o'-p') (arrows) and (m-n) v. (o-p) (between arrowheads), respectively. Note that for the ex vivo brains, images of the same specimens were taken at different time points, while for the in vivo controls different brains had to be fixed and imaged for the different time points. Scale bars, 20 μm.

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Figure 1—figure supplement 1. Culture imaging chamber. (a) Step-by-step construction of the imaging chamber. (i) Spacers are placed on the Sylgard layer in a triangle formation. (ii) A drop of diluted dialyzed agarose is pipetted onto the Sylgard. (iii) Dissected eye-brain complex is placed into the agarose drop. (iv) The mix is covered with a coverslip. (v) After the agarose polymerization, remaining space under coverslip is filled with the culture media; (vi) and sealed completely with rubber cement. The schematic of the final chamber (b) from the side and (c) the top.

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Figure 1—figure supplement 2. Brain development in imaging chamber compared to liquid media. Changes in brain morphology during development ex vivo in chamber vs. ex vivo in liquid media (free floating) vs. in vivo, from brains dissected at P + 24% (a-d), P + 50% (f-i) and cultured for 24 hr. Parallel developed in vivo controls (e, j) were dissected at the end of cultures. At early stages, brains that developed in the imaging chamber (b) are more similar in morphology to the in vivo controls (e) than the brains that developed in fully liquid media (d).
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Figure 2. Effects of culture conditions and laser scanning on the optic lobe development ex vivo. Two-photon imaging of the medulla was performed with brains cultured at P + 22% for 20 hr (a) and P + 41% for 19 hr (d) all photoreceptors express CD4-tdGFP. For each experiment one image stack was acquired containing both optic lobes of a brain. Next, only one of the lobes was scanned every 30 min. Finally, another stack was acquired with both lobes. Different brains aged in parallel in pupae have been dissected as in vivo controls. (b) Quantification of the layer distance increase in P + 22% cultures. The distance between R8 (green rectangles in a,d) and R7 (blue rectangles) layers increase identically in scanned and unscanned ex vivo lobes, but higher than the in vivo control (p = 0.0036, n = 3). (c) Quantification of the change in the angle between the planes of posterior lamina and the anterior medulla. Ex vivo lobes rotate similarly but slower than in vivo controls (p <0.0001, n = 3). (e) Quantification of the layer distance increase in P + 41% cultures. All groups show a similar increase in the distance between R8 temporary layer and R7 terminals. Error bars depict SEM. (f) Calibration of the developmental speed in culture to in vivo development, based on distal medulla expansion. Scale bars, 10 μm.

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Figure 2—figure supplement 1. Lamina rotation is incomplete ex vivo. Two-photon imaging of the medulla was performed with brains cultured at P + 22% for 20 hr, all photoreceptors express CD4-tdGFP. Continuously scanned ex vivo culture, unscanned control optic lobe and in vivo (fixed) control experiments were done as described in Figure 2. The angles (blue arches) between the planes of posterior lamina and anterior medulla have been measured for the start and end points of each culture as well as the corresponding in vivo controls, and plotted in Figure 2c. Scale bar, 10 μm.

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Figure 2—figure supplement 2. Effects of 20-Hydroxyecdysone (20-HE) and type of microscope on imaging in the culture chamber. (a-h) 20-HE is required for early but detrimental to late pupal development in the optic lobe. (a-d) All photoreceptors were labeled with CD4-tdGFP. Cultures were set-up at P + 22% (a), with (b) or without (c) 20-HE in the culture media. Parallel developed pupae were dissected and imaged at the end of cultures as in vivo controls (d). R7-R8 layer separation in the medulla was impaired in cultures without 20-HE compared to in vivo controls or cultures with 20-HE. Scale bars, 10 μm. (e-h) All photoreceptors were labeled with td-Tomato and R7 cells were sparsely labeled with CD4-tdGFP using GMR-FLP through MARCM. Cultures were set-up at P + 22% (e), with (f) or without (g) 20-HE in the culture media. Parallel developed pupae were dissected and imaged at the end of cultures as in vivo controls (h). R7 axons that developed in the presence of 20-HE showed excessive filopodial formations on their terminals compared in vivo controls or the cultures without 20-HE. Scale bars, 4 μm. (i) Comparison of resonant confocal and 2-photon microscopy signal strengths in the imaging culture chambers. R7 cells were sparsely labeled with CD4-tdGFP using GMR-FLP through MARCM. Individual R7 growth cones were imaged in the culture chamber at P + 30%. Images were acquired with a Leica TCS SP5 confocal microscope with a resonant scanner or a Zeiss LSM 780 multiphoton microscope at various depths from the coverslip. The confocal signal reduces below 60 μm compared to the 2-photon signal.

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Figure 3. Different filopodial signatures accompany separate circuit formation steps. Slow (30 min interval) time-lapse imaging of pupal brains dissected at P + 20% (a), P + 40% (b) and P + 55% (c) in comparison with in vivo fixed controls at the same stages. The same growth cones were analyzed for all live imaging experiments while different samples from parallel aged pupae had to be dissected for the in vivo controls. All photoreceptors were labeled with myr-mRFP and R7 cells were sparsely labeled with CD4-tdGFP using GMR-FLP through MARCM. (a) As the R7 and R8 layers go through their initial separation (upper panel), R7 terminals have numerous filopodia that invade neighboring columns (lower panel), which are pruned around P + 40% both ex vivo and in vivo. (b) As the layers start to reach their final configuration, R7 terminals form a bipartite structure around P + 50%. Filopodia numbers remain low. Around P + 55%, more (shorter) filopodia are observed again as R7 axon assumes a brush-like look. (c) After P + 55% shorter filopodia are pruned and R7 growth cones form new, longer filopodia that are fewer in number and have bulbous tips (arrows). Quantifications of (d) total number of filopodia per growth cone and (e), mean length of filopodia through the ex vivo experiments (a-c) and respective in vivo controls. Error bars depict SEM. Scale bars, 5 μm.

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Figure 3—figure supplement 1. Filopodial dynamics are restricted to the growth cone and axon shaft inside the medulla neuropil. (a) representative R7 terminal structures inside the medulla neuropil (grey background) reveal the transition of a more classical growth cone to a branched axonal structure. (b) 3D visualization of individual R7 axons (green) on the background of all photoreceptors (magenta) at P + 70%. (c) analysis of R7 axons and extended growth cones/axon shafts in the medulla reveals that filopodia only occur within the medulla neuropil.

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Figure 4. Distinct classes of transient and stable filopodia underlie different developmental events. Fast (1 min interval) time-lapse imaging was performed at multiple points of three ex vivo experiments. (a) Three time points are shown; during the first-stage (P + 28%) and second-stage (P + 50%) layer formation, and synaptogenesis (P + 60%). 3D graphs (upper panel) show the dynamics of individual filopodia observed in a one hour period. In the heat maps on blue background, individual filopodia are shown as vertical lines. The filopodia were sorted by their initial orientation angle (x-axis). The length of the vertical lines represents the life time of the filopodia (time on the y-axis). The color map indicates the length (µm) for each filopodium through time. Representative images of the growth cones at the above time-points (lower panel). See Figure 4—figure supplement 1 for heat maps and representative images at all time points. (b) Numbers of filopodia per growth cone for the time-points shown in a; for filopodia with lifetime <8 min (transient) and lifetime >1 hr (stable). (c) Numbers of filopodia relative to the numbers at P + 28% for all time-points imaged. Fitted curves: $y = 28.17 + 4.597x - 0.075x^2$ (transient) and $y = 583.1 - 24.53x + 0.26x^2$ (stable). (d) Mean length (µm) (e) Average speed (µm/min) and (f), Inactivity (ratio of intervals with no significant extension or retraction) for transient and stable filopodia at all time-points. Stable filopodia observed after P + 50% have significantly

Figure 4 continued on next page

Ozel et al. eLife 2015;4:e10721. DOI: 10.7554/eLife.10721
higher inactivity than those observed before (Means: 0.3002 v. 0.4346, p = 0.0002, n = 14 for each). See Figure 4—figure supplement 2 for these parameters as a function of filopodia lifetime on the same growth cone. Error bars depict SD. Scale bars, 2 μm.

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**Figure 4—figure supplement 1.** Fast filopodial dynamics throughout pupal development. Dynamics data from all 6 growth cones (2 independent growth cones for each time point) that were used in Figure 4. The heat maps on blue background show individual filopodia as vertical lines. The filopodia were sorted by their initial orientation angle (x-axis). The length of the vertical lines represents the lifetime of the filopodia (time on the y-axis). The images show the progression of filopodial dynamics across different time points and stages of development. The figure continues on the next page.
Figure 4—figure supplement 1 continued

axis). The color map indicates the length (μm) for each filopodium through time. a, starting at P + 28%, after 9 hr in culture and after 19 hr in culture. b, starting at P + 40%, after 8 hr in culture and after 21 hr in culture. (c), starting at P + 52% and 8 hr in culture. Scale bars, 3 μm.
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Figure 4—figure supplement 2. Filopodial dynamics as a function of lifetime. (a–i) For the same growth cones depicted in Figure 4, every filopodia observed in a 1 hr period were binned into different lifetime classes: <1 min, 2–3 min, 4–7 min, 8–15 min, 16–31 min, 32–59 min or >60 min. Mean length (a–c), speed (d–f) and inactivity (g–i) were plotted for each group of the three growth cones. The boxes cover the entire range and horizontal lines show the mean. Scale bars, 2 μm. (j–k), Mean inactivity (ratio of intervals with no significant extension or retraction) for transient (<8 min) and stable (>60 min) filopodia at all time-points. (j), Inactivity: due to the high ratio of filopodia with ‘zero’ inactivity in transient filopodia (g–i), average inactivity appear much lower for transient filopodia. (k) Inactivity of filopodia with at least one inactive time point: after the exclusion of ‘zero inactivity’ filopodia, inactivity of transient and early stage stable filopodia are identical. Error bars depict SD.

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**Figure 5.** R7 growth cones do not actively extend in the medulla. (a) R7 may reach its final target layer through active extension or passive displacement and intercalation. (b) Live imaging starting at P + 30%. All photoreceptors were labeled with myr-mRFP and R7 cells were sparsely labeled with CD4-tdGFP using GMR-FLP through MARCM. R7 growth cone (triangle) initially has a cone structure. As the layer formation progresses, a new varicosity (arrow) is formed from the axon shaft. This structure expands further and by P + 50% the entire terminal thickens. See Figure 5—figure supplement 1 for all time points. (N = 31). (c) Live imaging starting at P + 42%. Both R7 and R8 cells were sparsely labeled with CD4-tdGFP using hsFLP. R7 axon has already formed its distal varicosity (arrowhead); the R8 axon has extended a single filopodia proximally (arrow). Later, this filopodia reaches to the R8 final layer and forms the new terminal. R7 terminal shows no active extension activity. (N = 17 for R7 and 15 for R8). (d) Model of layer formation in the distal medulla. After their arrival to the medulla R7 and R8 terminals are initially separated by intercalation of lamina cell (LM) axons. After P + 40%, R8 growth cones actively extend to new layer while R7s remain in their arrival layer throughout. Scale bars, 3 µm.

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Single growth cone tracking demonstrates R7 terminals remain passive throughout layer formation without a stationary landmark. Live imaging starting at P + 30%. All photoreceptors were labeled with myr-mRFP and R7 cells were sparsely labeled with CD4-tdGFP using GMR-FLP through MARCM. R7 terminal (red arrow) can be followed throughout 17.5 hr based on its specific filopodial morphology and dynamics. A new varicosity (yellow arrow) was formed from the axon shaft and expands over the course of 15 hr, pushing the terminal distally. No directed activity was observed at the growth cone tip throughout the imaging period. Scale bar, 5 μm.

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Figure 6. N-Cadherin is required for the stabilization but not the layer specific targeting of R7 growth cones. All photoreceptors were labeled with myr-mRFP. CadND405 R7 cells were generated with MARCM, using GMR-FLP and positively labeled with CD4-tdGFP. (a) Live imaging started at P + 24% shows a mutant R7 growth cone (arrow) that retracts from its target layer over the course of 5 hr. (b), Live imaging started at P + 53% shows a mutant R7 growth cone (arrow) that retracts from its target layer over the course of 10 hr. Some mutant axons retract completely from the medulla (Figure 6—figure supplement 1) (c) Live imaging started at P + 48% shows an R7 axon (arrow) that has been retracted to the edge of distal medulla but re-extends and attempts to re-innervate both wrong (5.5 hr) and the right (7 hr) layers. (d) Schematics of observed retraction and re-extensions events. Left and middle: Full Retraction leads to complete loss of the R7 axons from the medulla (left), while partial retraction (middle) leads to R7 terminals in an incorrect layer. Number of mislocalized terminals: 33% (n = 85) at P + 40% and 56% (n = 62) at P + 52%. Right: Previously retracted R7 axons can re-extend, even days after they would have been stabilized in wild type. 52% (n = 23) of retracted axons at P + 40% re-extended before P + 50%. Scale bars, 5 μm.

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**Figure 6—figure supplement 1.** CadN mutant R7 axons may retract completely from the medulla. All photoreceptors were labeled with myr-mRFP. CadN^G05^ R7 cells were generated with MARCM, using GMR-FLP and positively labeled with CD4-tdGFP. Live imaging starting at P + 35% demonstrates an R7 axon which retracts from its target layer in the first 2 hr. During the remaining 7 hr, the axon retracts below the R8 temporary layer (upper red layer) and leaves the distal medulla completely. Scale bar, 4 μm.

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Figure 7. N-Cadherin is required for fast filopodial dynamics. CadN\textsuperscript{405} R7 cells were generated with MARCM, using GMR-FLP and positively labeled with CD4-tdGFP. Fast (1 min interval) time-lapse imaging was performed at P + 28%. (a), The average numbers of filopodia per growth cone are not significantly different between wt and CadN\textsuperscript{405}. (b) Mutant filopodia are slower, (for transient, means wt: 1.303 (n = 143), CadN\textsuperscript{405}, 0.791 (n = 169), p<0.0001; for stable, means wt: 0.898 (n = 10), CadN\textsuperscript{405} 0.636 (n = 5) p = 0.0199) and (c) shorter (for transient, means wt: 1.542 (n = 143), CadN\textsuperscript{405} 0.939 (n = 169), p <0.0001; for stable: means wt: 3.707 (n = 10), CadN\textsuperscript{405} 2.275 (n = 5), p = 0.1257). (d) CadN\textsuperscript{405} R7 growth cones at the correct layer. Scale bars, 5 μm.

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