Axonal chemokine-like Orion induces astrocyte infiltration and engulfment during mushroom body neuronal remodeling

Ana Boulanger¹,⁵✉, CamilleThinat¹, Stephan Züchner², Lee G. Fradkin³, Hugues Lortat-Jacob⁴ & Jean-Maurice Dura¹,⁵✉

The remodeling of neurons is a conserved fundamental mechanism underlying nervous system maturation and function. Astrocytes can clear neuronal debris and they have an active role in neuronal remodeling. Developmental axon pruning of Drosophila memory center neurons occurs via a degenerative process mediated by infiltrating astrocytes. However, how astrocytes are recruited to the axons during brain development is unclear. Using an unbiased screen, we identify the gene requirement of orion, encoding for a chemokine-like protein, in the developing mushroom bodies. Functional analysis shows that Orion is necessary for both axonal pruning and removal of axonal debris. Orion performs its functions extracellularly and bears some features common to chemokines, a family of chemoattractant cytokines. We propose that Orion is a neuronal signal that elicits astrocyte infiltration and astrocyte-driven axonal engulfment required during neuronal remodeling in the Drosophila developing brain.

¹IGH, Centre National de la Recherche Scientifique, Univ Montpellier, Montpellier, France. ²Dr. John T. Macdonald Foundation Department of Human Genetics and John P. Hussman Institute for Human Genomics, University of Miami, Miami, FL, USA. ³Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA. ⁴Institut de Biologie Structurale, UMR 5075, University Grenoble Alpes, Centre National de la Recherche Scientifique, Commissariat à l’Énergie Atomique et aux Énergies Alternatives, Université Grenoble Alpes, Grenoble, France. ⁵These authors jointly supervised this work: Ana Boulanger, Jean-Maurice Dura. ✉email: ana.boulanger@igh.cnrs.fr; jean-maurice.dura@igh.cnrs.fr
Neuronal remodeling is a widely used developmental mechanism, across the animal kingdom, to refine dendrite and axon targeting necessary for the maturation of neural circuits. Importantly, similar molecular and cellular events can occur during neurodevelopmental disorders or after nervous system injury1–4. A key role for glial cells in synaptic pruning and critical signaling pathways between glia and neurons have been identified5. In Drosophila, the mushroom body (MB), a brain memory center, is remodeled at metamorphosis and MB γ neuron pruning occurs by a degenerative mechanism6–8. Astrocytes surrounding the MB have an active role in the process; blocking their infiltration into the MBs prevents remodeling9,10, MB γ neuron remodeling relies on two processes: axon fragmentation and the subsequent clearance of axonal debris. Importantly, it has been shown that astrocytes are involved in these two processes and that these two processes can be decoupled12. Altering the edcsysone signaling in astrocytes, during metamorphosis, results both in a partial axon pruning defect, visualized as either some individual larval axons or as thin bundles of intact larval axons remaining in the adults, and also in a strong defect in clearance of debris, visualized by the presence of clusters of axonal debris. Astrocytes have only a minor role in axon severing as evidenced by the observation that most of the MB γ axons are correctly pruned when edcsysone signaling is altered in these cells. When astrocyte function is blocked, the γ axon-intrinsic fragmentation process remains functional and the majority of axons degenerate.

It has been widely proposed that a “find-me/eat-me” signal emanating from the degenerating γ neurons is necessary for astrocyte infiltration and engulfment of the degenerated larval axons7,9,13. However, the nature of this glial recruitment signal is unclear.

Here, we have identified a gene (orion), not previously described, by screening for viable ethyl methanesulfonate (EMS)-induced mutations and not for lethal mutations in MB clones as was done previously14,15. This allowed the identification of genes involved in glial cell function by directly screening for defects in MB axon pruning. We found that orion1, a viable X-chromosome mutation, is necessary for both the pruning of some γ axons and removal of the resulting debris. We show that Orion is secreted from the neurons, remains near the axon membranes where it associates with infiltrating astrocytes, and is necessary for astrocyte infiltration into the γ bundle. This implies a role for an as-yet-undefined Orion receptor on the surface of the astrocytes. Orion bears some chemokine features, for example, a CX3C motif, three glycosaminoglycan (GAG) binding consensus sequences that are required for its function. Altogether, our results identify a neuron-secreted extracellular messenger, which is likely to be the long-searched-for signal responsible for astrocyte infiltration and engulfment of the degenerated larval axons and demonstrate its involvement for neuronal remodeling.

Results and discussion

The orion gene is necessary for MB remodeling. Adult orion1 individuals showed a clear and highly penetrant MB axon pruning phenotype as revealed by the presence of some adult unpruned vertical γ axons as well as the strong presence of debris (100% of mutant MBs; n = 100) (Fig. 1a, b, Table 1, and Supplementary Figs. 1 and 2). Astrocytes, visualized with alrm-GAL4, are the major glial subtype responsible for the clearance of the MB axon debris12. The presence of γ axon debris is a landmark of defective astrocyte function, as was described11,12, and is also further shown in this study (Supplementary Fig. 1a–d). The unpruned axon phenotype was particularly apparent during metamorphosis (Fig. 1c–h). At 24 h after pupariation formation (APF), although γ axon branches were nearly completely absent in the wild-type control, they persisted in the orion1 mutant brains, where we also observed a significant accumulation of debris (Fig. 1e, h). The number of unpruned axons at this stage is lower in orion1 than in Hr39f,12 where the γ axon-intrinsic process of pruning is blocked (Supplementary Fig. 1e–g). In addition, the MB dendrite pruning was clearly affected in orion1 individuals (Supplementary Fig. 1h–p).

The orion gene encodes for a CX3C motif-containing secreted proteins. The orion1 EMS mutation was localized by standard duplication and deficiency mapping as well as by whole-genome sequencing (Fig. 2a). The orion gene (CG2206) encodes two putatively secreted proteins: Orion-A (664 amino acid (a.a.)) and Orion-B (646 a.a.), whose messenger RNAs (mRNAs) arise from two different promoters (Fig. 2b–d). These two proteins differ in their N-terminal domains and are identical in the remainder of their sequences. The EMS mutation is a G to C nucleotide change inducing the substitution of the glycine (at position 629 for Orion-A and 611 for Orion-B) into an aspartic acid. The mutation lies in the common shared part and therefore affects both Orion-A and -B functions. Both isoforms display a signal peptide at their N termini, suggesting that they are secreted. Interestingly, a CX3C chemokine signature is present in the Orion common region (Fig. 2b, c). Chemokines are a family of chemoattractant cytokines, characterized by a CC, CXC, or CX3C motif, promoting the directional migration of cells within different tissues. Mammalian CX3CL1 (also known as fractalkine) is involved in, among other contexts, neuron–glia communication16–20. Mammalian Fractalkines display conserved intramolecular disulfide bonds that appear to be conserved with respect to their distance from the CX3C motif present in both Orion isoforms (Fig. 2c). Fractalkine and its receptor, CX3CR1, have been recently shown to be required for post-trauma cortical brain neuron microglia-mediated remodeling in a mouse whisker lesioning paradigm21. We observed that the change of the CX3C motif into CX4C blocked the Orion function necessary for the MB pruning (Supplementary Fig. 3a–c, h–j). Similarly, the removal of the signal peptide also prevented pruning (Supplementary Fig. 3d, h–j). These two results indicate that the Orion isoforms likely act as secreted chemokine-like molecules. We also produced three CRISPR/Cas9-mediated mutations in the orion gene, which either delete the common part (orionΔC), the A-specific part (orionΔA), or the B-specific part (orionΔB). Noticeably, orionΔC displayed the same MB pruning phenotype as orion1, which is also the same in orion1/Deficiency females, indicating that orion1 and orionΔC are likely null alleles for this phenotype. In contrast, orionΔA and orionΔB have no MB phenotype by themselves indicating the likelihood of functional redundancy between the two proteins in the pruning process (Supplementary Fig. 4).
Fig. 1 The *orion* gene is necessary for MB remodeling. a–l γ neurons are visualized by the expression of 201Y-GAL4-driven UAS-mCD8-GFP (green). In adults, this GAL4 line also labels the αβ-core axons shown here by asterisks. a, b Adult γ axons in control (a) and *orion*¹ (b). Note the presence of unpruned γ axon bundles (arrowhead) and the high amount of uncleared axonal debris (arrows) in *orion*¹ compared to wild-type. (n ≥ 100 MBs for control and *orion*¹. See quantitation in Table 1 and Supplementary Fig. 2.) c–h γ axon development in wild-type (c–e) and *orion*¹ (f–h) at 6, 18, and 24 h APF as indicated. Unpruned axons (arrowhead) in *orion*¹ are already apparent at 18 h APF (compare g with d) although no differences are detected at 6 h APF (c, f). Note the presence of unpruned γ axons (arrowhead) and debris (arrow) in *orion*¹ at 24 h APF (n = 40 MBs for each developmental stage). i–k The adult *orion*¹ phenotype (i) is completely rescued by expression in MBs of UAS-orion-A (n = 89 MBs) (j) or UAS-orion-B (n = 387 MBs) (k). l UAS-orion-RNAi expression in MBs results in unpruned γ axon phenotypes (arrowheads) (n = 20 MBs). Scale bars represent 40 μm. All the images are composite confocal images. Genotypes are listed in the Supplementary list of fly strains.
produced unpruned axons similar to that in orion1, although the debris is not apparent likely due to an incomplete inactivation of the gene expression by the RNAi (Fig. 11 and Supplementary Fig. 3d). The expression of the same RNAi in the glia had no effect (Supplementary Fig. 5e). Using the mosaic analysis with a repressible cell marker (MARCM23), we found that orion homozygous mutant neuroblast clones of γ neurons, in orion1/γ phenotypically wild-type individuals, were normally pruned (Supplementary Fig. 6a, b). Therefore, orion1 is a non-cell-autonomous mutation that is expected if the Orion proteins are secreted. Orion proteins secreted by the surrounding wild-type neurons is transcribed at 0 h APF and dramatically decreases at 9 h APF with a peak at 3 h APF, as a likely transcriptional target of EcR-B1 and Sox1424 and this is also consistent with earlier microarray analysis observations 25. Noticeably, forced expression of UAS-EcR-B1 orion RNAi and drprΔS in the MB nuclei, is not altered in orion1 individuals (Supplementary Fig. 6c, f). Furthermore, the unpruned axon phenotype produced by orion RNAi is rescued by forced expression of EcR-B1 in the MBs (Supplementary Fig. 3h). Therefore, our genetic interaction analyses support Orion being downstream of EcR-B1.

**Orion is required for the infiltration of astrocytes into the MB y bundle.** Since glial cells are likely directly involved in the orion1 pruning phenotype, we examined their behavior early during the pruning process. At 6 h APF the axon pruning process starts and is complete by 24 h APF, but the presence of glial cells in the vicinity of the wild-type γ lobes is already clearly apparent at 6 h APF9. We examined glial cells visualized by a membrane-targeted GFP (UAS-mGFP) under the control of repo-GAL4 and stained the γ axons with anti-Fas2. At 6 h APF, a striking difference was noted between wild-type and orion1 brains. Unlike in the wild-type control, there is essentially no glial cell invasion of the γ bundle in the mutant (Fig. 4a–c). Interestingly, glial infiltration as well as engulfment of the degenerated larval axons was not observed in orion1 neither at 12 h APF nor at 24 h APF (Supplementary Fig. 8a–h), suggesting that glial cells never infiltrate MBs in mutant individuals. We also ruled out the possibility that this lack of glial cell activity was due to a lower number of astrocytes in mutant versus wild-type brains (Supplementary Fig. 8i, j).

We also examined the proximity between MB Orion-Myc and astrocytes, as inferred from the shape of the glial cells, labeled with the anti-Drpr antibody at 6 h APF (Fig. 4d–f). We looked at the distribution along the vertical γ lobes (60 µm of distance, see “Methods”) of Orion-B–Myc (wild-type protein) and of Orion-B–ΔSP–Myc (not secreted), in an otherwise wild-type background. We quantified only from images where an astrocyte sat on the top of the vertical lobe. A peak of Orion-Myc localization was always found (n = 10) in the axonal region close to the astrocyte (<7 μm) when wild-type Orion-B–Myc was quantified (Fig. 4g, i). However, this was not the case (n = 9) when Orion-B–ΔSP–Myc was quantified (Fig. 4h, j). This strongly suggests that astrocytic processes may be “attracted” by secreted Orion.

| Table 1 Unpruned axon and axon debris quantitation. |
|---------------------------------------------|
| (a) Presence of unpruned axons in ≥1-week-old adults |
|                | MB | None | Weak | Strong |
| WT              | 25 | 25   | 0    | 0      |
| Hr39            | 22 | 0    | 22   | 0      |
| orionΔC         | 22 | 0    | 22   | 0      |
| orion1          | 20 | 0    | 20   | 0      |
| orion RNAi      | 34 | 0    | 34   | 0      |
| drprΔS          | 22 | 20   | 2    | 0      |

| (b) Presence of axon debris in ≥1-week-old adults |
|---------------------------------------------|
|                | MB | None | Mild | Intermediate | Strong |
| WT              | 25 | 25   | 0    | 0            | 0      |
| Hr39            | 22 | 22   | 0    | 0            | 0      |
| orionΔC         | 22 | 0    | 0    | 22           | 0      |
| orion1          | 20 | 0    | 20   | 0            | 0      |
| orion RNAi      | 34 | 34   | 0    | 0            | 0      |
| drprΔS          | 22 | 16   | 2    | 2            | 2      |

| (c) Presence of axon debris in ≤2-h-old adults |
|---------------------------------------------|
|                | MB | Scattered dots | Mild | Intermediate | Strong |
| WT              | 10 | 10             | 0    | 0            | 0      |
| orionΔC         | 12 | 0              | 0    | 0            | 12     |
| drprΔS          | 73 | 40             | 11   | 4            | 18     |

Genotypes are indicated on the left. “MB” indicates the number of mushroom bodies observed for each genotype. Unpruned axons were ranked into three categories: “None” indicates the absence of unpruned γ axons and “Weak” and “Strong” refers to different levels of the mutant pruning phenotype. Axon debris were ranked into five categories: “None” indicates the absence of debris and “Scattered dots” means that some individual debris can be observed. “Mild,” “Intermediate,” and “Strong” refer to different levels of debris (see Supplementary Fig. 2 and "Methods"). Full genotypes are listed in the Supplementary list of fly strains.
Moreover, we observed that extracellularly present Orion stays close to axon membranes (Supplementary Fig. 9a–f). Protein, in particular chemokine, localization to membranes is often mediated by GAGs, a family of highly anionic polysaccharides that occur both at the cell surface and within the extracellular matrix. GAGs, to which all chemokines bind, ensure that these signaling proteins are presented at the correct site and time in order to mediate their functions. We identified three consensus sequences for GAG linkage in the common part of Orion (Fig. 2d). We mutated these sequences individually and assayed the mutant proteins for their ability to rescue the orion1 pruning defect in vivo. The three GAG sites are required for full Orion expression.
function, although mutating only GAG3 produced a strong mutant phenotype (Supplementary Fig. 3e–j).

Our findings imply a role for an as-yet-undefined Orion receptor on the surface of the glial cells. The glial receptor draper (drpr) seemed an obvious candidate, although Drpr ligands unrelated to Orion have been identified. The MB remodeling phenotypes in Orion mutant or drpr are, however, different from Orion mutant phenotype being stronger than the drpr one. The use of an UAS-mGFP driven by 201Y-GALA4, instead of anti-Fas2, where the labeling of αβ axons often masks individual γ axons, allowed us to observe occasionally unpruned axons in drpr 1-week-old post-eclosion brains in addition to uncleaved debris (Table 1 and Supplementary Fig. 2), indicating a certain degree of previously undescribed unpruned axon persistence in the mutant background. Nevertheless, only Orion mutant displayed a 100% penetrant phenotype of both unpruned axons and debris (strong category) in adult flies, which are still present in old flies. On the contrary, the weaker drpr mutant phenotype strongly decreases throughout adulthood (Table 1 and Supplementary Fig. 1). This suggests that Drpr is not, or at least not the sole, Orion receptor.

Independently of the possible role of Drpr as an Orion receptor, we wished to test if Orion could activate the drpr signaling pathway as it is the case for neuron-derived injury released factors and Spätzle ligands, which bind to glial insulin-like receptors and Toll-6, respectively, upregulating in turn the expression of drpr in phagocytic glia. These ligands are necessary for axonal debris elimination and act as a find-me/eat-me signals in injury and apoptosis as Orion is doing for MB pruning. Our data indicate that Orion does not modify neither the Drpr expression nor the level of the drpr transcriptional activator STAT92E in astrocytes. Consequently, Orion does not seem to induce the Drpr signaling pathway in astrocytes.

We have uncovered a neuronally secreted chemokine-like protein acting as a find-me/eat-me signal involved in the neuron–glia crosstalk required for axon pruning during developmental neuron remodeling. To the best of our knowledge, chemokine-like signaling in insects was not described previously and, furthermore, our results point to an unexpected conservation of chemokine CX3C signaling in the modulation of neural circuits. Thus, it is possible that chemokine involvement in neuron/glial cell interaction is an evolutionarily ancient mechanism.

**Methods**

**Drosophila stocks.** All crosses were performed using standard culture medium at 25°C. Except where otherwise stated, alleles have been described (http://flystocks.bio.indiana.edu). The following alleles were used: orion1, orion4A, orion5B, and orionAC were generated in this study. drprGal4 was found to have an unrelated lethal mutation, which was removed by standard mitotic recombination over a wild-type chromosome. Animals bearing this version of drprGal4 survive to adult stages and were used for this work. The following UAS lines were used for this work: UAS-orion-RNAi (VDRC stock 30843) and 2x UAS-drpr-myc, 10X-Stat92E-GFP, UAS-orion-A, UAS-orion-A-myc, UAS-orion-B, UAS-orion-B-myc, UAS-orion-B-Mut AX3C-myc, UAS-orion-B-Mut CX4C-myc, UAS-orion-B-ASp-myc, UAS-orion-B-Mut GAG1-myc, UAS-orion-B-Mut GAG2-myc, and UAS-orion-B-Mut GAG3-myc were generated in this study. We used three GAL4 lines: 201Y-GALA4 expressed in MB neurons, alrm-GALA4 expressed in glia astrocytes and the pan-glial driver repo-GALA4 expressed in all glia.

**Mutagenesis and screening.** EMS mutagenesis was carried out following the published procedure. EMS treated y w6723 snf FRT19A males were crossed to FM7c/p6w females and stocks, coming from single y w6723 snf FRT19A/ FM7c female crossed to FM7c males, were generated. Only viable y w6723 snf FRT19A chromosome bearing stocks were kept and y w6723 snf FRT19A; UAS-mCD8-GFP 201Y-GALA4+/ adult males from each stock were screened for MB neuronal remodeling defect with an epi-fluorescence microscope (Leica DM 6000).

**Mapping of Orion.** To broadly map the location of the EMS-induced mutation on the X-chromosome, we used males from the stocks described in the X-chromosome duplication kit (Bloomington Stock Center) that we crossed with orion1; UAS-mCD8-GFP 201Y-GALA4 females. Dp(1;Y)BSC346 (stock 36487) completely rescued the orion1 y axon unpruned phenotype. This duplication is located at 6D5–6E2; 7D18 on the X chromosome. We then used smaller duplications covering this region. Thus, duplications Dp(1;3)DC946 (stock 33489) and Dp(1;3)DC183 (stock 32271) also rescued the orion1 mutant phenotype. However, duplication Dp(1;3)DC184 (stock 30312) did not rescue the mutant phenotype. Overlapping of duplications indicates that the EMS mutation was located between 7C9 and 7D2, which comprises 72 kb. In addition, deficiency Def(1)C128 (stock 949, Bloomington Stock Center), which expand from 7D1 to 7D5-D6, complements orion1 contrary to deficiency Def(1)BSC622 (stock 25697, Bloomington Stock Center), which does not (see Fig. 2a).

**Whole-genome sequencing.** Gene mutation responsible for the unpruned y axon phenotype was precisely located through the application of next-generation sequencing. The genomic DNA was extracted from 30 adult females (mutant and control) and directly sequenced on a HiSeq2000 next-generation sequencing platform (Illumina). Bioinformatics analysis for read alignment and variant investigation was carried out through the duplication mapping (see above) at the University of Miami Miller School of Medicine, Center for Genome Technology.

**Signal peptide and transmembrane protein domain research.** For prediction of signal peptide sequences, we used the PrediSi website (http://www.predisi.de; for transmembrane domains, we used the TMHMM Server, v 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).
GFP / anti-Myc

**Fig. 3 Orion is extracellularly present on MB γ axons.** a–k Six-hour APF γ axons are visualized by the expression of 201Y-GAL4-driven UAS-mCD8-GFP (green). a, b, j, k γ axons expressing the wild-type Orion-B-Myc protein (red) (n = 10 MBs). c γ axons expressing the Orion-B-Myc protein lacking the signal peptide (ΔSP) (n = 9 MBs). a–c are confocal Z-projections and j is a unique confocal plane. d, g Higher magnification images of the region indicated by a rectangle shows a representative unique confocal plane. Note the presence of Myc-labeled Orion-B outside the γ axon bundle (arrows). e, h Higher magnification images of the region indicated by a rectangle in b showing a representative unique confocal plane. Note the presence of Myc-labeled Orion-B inside the hole-like structures present in the γ axon bundle (arrowheads). f, i Higher magnification images of the vertical and medial γ lobes, respectively (rectangles in c). Orion-B-ΔSP-Myc is observed neither outside the γ axons (f) nor in the hole-like structures (arrowheads in i). j, k Presence of Myc-labeled Orion-B extracellular proteins not associated with GFP-labeled axon membranes can be observed outside the γ axon bundle (arrows). k Three-dimensional surface-rendering (3D) of the confocal image. j Reveals close apposition of GFP-labeled axons and Myc-labeled Orion and reveals Orion is present as small extracellular globules. Scale bars represent 40 μm in a–c, 20 μm in d–i and 5 μm in j, k. Full genotypes are listed in the Supplementary list of fly strains.
were aligned using the AlignX plug-in in the VectorNTi software package (Invitrogen) without permitting the introduction of spaces or deletions.

**GAG binding site research.** Identification of GAG binding sites in proteins, in the absence of structural data, is complicated by the diversity of both GAG structure and GAG binding proteins. Previous work based on heparin-binding protein sequence comparisons led to the proposition of two GAG binding consensus sequences, the XBBXBX and XBBBXXBX motifs (where B and X stand for basic and neutral/hydrophobic amino acids, respectively). A number of closely related basic clusters, including XBBXBXBX, were next experimentally identified. Visual examination of the Orion-B sequences returned three such clusters (XBBXXB XBBXBX; residues 242–254; XBBXBX; residues 416–421; and XBBXBXBX: residues 547–554, numbering includes the peptide signal, see Fig. 2d), which are also present in Orion-A.

**CRISPR-Cas9 strategy.** All guide RNA sequences (single guide RNA (sgRNA)) were selected using the algorithm targetfinder.flycrispr.neuro.brown.edu/ containing 20 nucleotides each (PAM excluded) and are predicted to have zero off-targets. We selected three pairs of sgRNA. Each pair is targeting either the A-specific region of Orion, the B-specific region of Orion, or the C common region of the two
Fig. 4 Orion is required for the infiltration of astrocytes into the MB γ bundle and engulfment of the larval axons at 6 h APF. a–c Single confocal planes of 6 h APF brains expressing repo-GAL4-driven UAS-mCD8-GFP (green) in controls (a, b) and orion1 (c) focused on the MB dorsal lobe (n = 12 MBs controls and n = 20 MBs orion1). Anti-Fas2 staining (red) reveals spherical hole-like structures occupied by glial processes infiltrating into the γ bundle (green, arrows) in wild-type (a, b), but not in orion1 individuals (c). Scale bars are 20 μm. d–f A single confocal plane showing the expression of 20Y14-GAL4-driven UAS-mCD8-GFP (green, d–f) and Orion-B-Myc (red, d–f) in 6 h APF MB. Anti-Drpr antibody (blue) was used to visualize the glial cells (blue, e, f). d–f display the same MB. d displays Orion-B-Myc expression outside the axons at the top of the vertical γ bundle (arrows) as well as in hole-like structures (arrowheads). e displays an astrocyte positioned at the top of the γ bundle (asterisk in its nucleus) as well as several astrocyte processes occupying hole-like structures (arrowheads). Note the colocalization of Orion-B-Myc and glia processes in the hole-like structures (arrowheads in f). The astrocyte cell membrane (continuous line) and the membrane contacting the tip of the γ bundle (dotted line), where phagocytosis is taking place, based on our interpretation of the astrocyte limits according to the green and the blue channels for GFP and Drpr, respectively, are indicated in figure settings were constants. However, since the signal of unpruned axons is estimated by the width of the corresponding medial bundle compared with the width of the medial pruned axon bundle, for debris analysis, we established five categories: none, scattered dots, mild, intermediary, and strong based on the location and size of the debris clusters13. In 21–24 h-old adults, “none” means the absence of debris. In 2–2 h-old adults “scattered dots” means some individual debris can be observed. We considered “mild,” if debris clusters (clusters >5 μm2) appear only at one location, “intermediary,” at two locations, and “strong,” at three locations of the MB. The three debris locations were considered: the tip of the vertical lobe, the tip of the medial lobe, and around the heel (bifurcation site of γ axons into dorsal and medial). For EcR-B1 signal quantitation, we performed five measurements for each picture (intensity 1, …, 5) in the nuclei of GFP-positive cell bodies and the same number of measurements in the background using confocal single slices. The mean of the five background measurements was calculated as mean background. We then calculated the signal intensities of mean background from each intensity value (intensity 1, …, 5 minus mean background) to obtain normalized intensity values. Finally, we compared normalized intensity values between two genetic conditions. We proceeded similarly for Draper and STAT-GFP signal quantitation, but staining was quantified in the astrocyte cytoplasm located in the immediate vicinity of the γ dorsal lobe. Quantitation of intensity was performed using the ImageJ software. To quantify the Myc signal in the γ vertical lobe, we traced a 60 μm line on the Cy3 red Z-stack and used the Plot Profile function of ImageJ to create a plot of intensity across the line. The tip of the line (μm) was the tip of the γ vertical lobe and the bottom of the line (60 μm) at the branching point of the two γ lobes. Only images containing an astrocyte sitting at the top of the γ vertical lobe were used to quantify Myc expression levels in Orion-B- and Orion-B-ΔSP-expressing MBs. To quantify the number of astrocytes around the γ lobes, we counted the number of glial nuclei, as labeled with anti-Repo antibody, contained in GFP-positive astrocyte cytoplasm labeled with UAS-mCD8-GFP driven by abram-GAL4. We only counted nuclei contained within a circle of 70 μm of diameter centered in the middle of the vertical γ lobe tips.

UAS constructs. The Orion-A cDNA (complementary DNA) inserted in the pPT2 plasmid (clone LD24308) was obtained from Berkeley Drosophila Genome Project (BDGP). Initial Orion-B cDNA as well as the Orion-B cDNAs containing mutations or orion1 were lacking the γ-spg domain and the GAG signal peptide. We used an Orim construct (Piscataway, NJ) in the pcdNA3.1-C-(k)DYK vector. The Orion-B cDNAs contained the following mutations:

1. To remove the signal peptide, we removed the sequence: CAGCGCGGCTTCGAGATTATTCGCTGCCTTGGTTCGCTCTGCACTGCT TGGATTTTGTTGAATAA AATGTCG TGC. 2. C. The level of expression increased in this particular case in order to get similar levels than in Orion-B-Myc. We used the Imaris (Bitplane) software to generate a pseudo 3D structure of Orion-produced γ-axons (Imaris surface tool). We created two 3D surfaces, from regular confocal images, defining the axonal domain (green) and the 3D expression domain (red).

Quantification of immunolabelling. To quantify unpruned γ axons, we established three categories of phenotypes: “none,” when unpruned axons are observed, “weak,” when few unpruned individual axons or thin axon bundles are observed in the dorsal lobe, and “strong,” when >50% of the axons are unpruned. In this last category, the percentage of unpruned axons is estimated by the width of the corresponding medial bundle compared with the width of the medial pruned axon bundle. For debris analysis, we established five categories: none, scattered dots, mild, intermediary, and strong based on the location and size of the debris clusters13. In 21–24 h-old adults, “none” means the absence of debris. In 2–2 h-old adults “scattered dots” means some individual debris can be observed. We considered “mild,” if debris clusters (clusters >5 μm2) appear only at one location, “intermediary,” at two locations, and “strong,” at three locations of the MB. The three debris locations were considered: the tip of the vertical lobe, the tip of the medial lobe, and around the heel (bifurcation site of γ axons into dorsal and medial).

ToEcR-B1 signal quantitation, we performed five measurements for each picture (intensity 1, …, 5) in the nuclei of GFP-positive cell bodies and the same number of measurements in the background using confocal single slices. The mean of the five background measurements was calculated as mean background. We then calculated the signal intensities of mean background from each intensity value (intensity 1, …, 5 minus mean background) to obtain normalized intensity values. Finally, we compared normalized intensity values between two genetic conditions. We proceeded similarly for Draper and STAT-GFP signal quantitation, but staining was quantified in the astrocyte cytoplasm located in the immediate vicinity of the γ dorsal lobe. Quantitation of intensity was performed using the ImageJ software. To quantify the Myc signal in the γ vertical lobe, we traced a 60 μm line on the Cy3 red Z-stack and used the Plot Profile function of ImageJ to create a plot of intensity across the line. The tip of the line (μm) was the tip of the γ vertical lobe and the bottom of the line (60 μm) at the branching point of the two γ lobes. Only images containing an astrocyte sitting at the top of the γ vertical lobe were used to quantify Myc expression levels in Orion-B- and Orion-B-ΔSP-expressing MBs. To quantify the number of astrocytes around the γ lobes, we counted the number of glial nuclei, as labeled with anti-Repo antibody, contained in GFP-positive astrocyte cytoplasm labeled with UAS-mCD8-GFP driven by abram-GAL4. We only counted nuclei contained within a circle of 70 μm of diameter centered in the middle of the vertical γ lobe tips.

Microscopy and image processing. Images were acquired at room temperature using a Zeiss LSM 780 laser scanning confocal microscope (MRI Platform, Institute of Human Genetics, Montpellier, France) equipped with a ×40 PLAN apochro- matic oil immersion differential interference contrast objective lens. The immersion oil used was Immersol 518F. The acquisition software used was Zen 2011 (black edition). Contrast and relative intensities of the green (GFP), of the red (Cy3), and of the blue (Cy5 and Alexa 647) channels were processed with the ImageJ and Fiji software. Settings were optimized for detection without saturating the signal. For each set of five settings, the constants were: 500, 1:300 for detection without saturating the signal. For each set of five settings, the constants were: 500, 1:300 for detection without saturating the signal.
orion-AB rev (see the corresponding oligonucleotide sequences in Supplementary Table 1).

Amplified cDNA was processed for pENTR/D-TOPO cloning (Thermo Fisher Scientific, K240020) and constructs were subsequently sequenced (Geneviz, France). We used the Gateway LR clonase enzyme mix (Thermo Fisher Scientific, 11791019) to recombine the inserts into the destination UAS vector pJFRC81-GW-6. Yu, F. & Schuldiner, O. Axon and dendrite pruning in Drosophila. Cell 9. Neukomm, L. J. & Freeman, M. R. Diverse cellular and molecular modes of neuronal remodeling in Drosophila. Neuron 28, 407–815 (2000).

Zhang, X. et al. TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the Drosophila brain. Cell 112, 303–305 (2003).

Paolicelli, R. C., Bisti, K. & Tremblay, M. E. Fractalkine regulation of microglial physiology and consequences on the brain and behavior. Front. Cell. Neurosci. 8, 129 (2014).

Arnaux, I. & Audinat, E. Fractalkine signaling and microglia functions in the developing brain. Neur. 2015, 68940 (2015).

Wernburg, N., Seinberg, P. A., Johnson, K. M. & Schaefer, D. P. A microglia–cytokine axis to modulate synaptic connectivity and function. Curr. Opt. Neurobiol. 47, 138–145 (2017).

Luo, P., Chu, S. F., Zhang, Z., Xia, C. Y. & Chen, N. H. Fractalkine/CX3CR1 is involved in the cross-talk between neuron and glia in neurological diseases. Brain Res. Bull. 146, 12–21 (2019).

Wilson, D. K., Dissing-Olesen, L. & Stevens, B. Neuron-glia signaling in synapse elimination. Annu. Rev. Neurosci. 42, 107–127 (2019).

Lehman, G. et al. Sensory lesioning induces microglial synapse elimination via ADAM10 and fractalkine signaling. Nat. Neurosci. 22, 1075–1088 (2019).

Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 110, 401–415 (1993).

Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker for studies of neuronal morphology in neuronal morphogenesis. Neuron 22, 451–461 (1998).

Aloy, I. et al. Combining developmental and perturbation-seq uncoovers transcriptional modules orchestrating neuronal remodeling. Dev. Cell 47, 38–52 e36 (2018).

Hoopfer, E. D., Penton, A., Watts, R. J. & Luo, L. Genomic analysis of Drosophila mushroom body axon pruning. Dev. Cell 28, 609–618 (2010).

Reynaud, E. et al. Guidance of Drosophila mushroom body axons depends upon DRL-Wnt receptor cleavage in the brain dorsomedial lineage precursors. Cell Rep. 11, 1293–1304 (2015).

Thomas, R. E. et al. Glucocerebrosidase deficiency promotes protein aggregation through dysregulation of extracellular vesicles. PLoS Genet. 14, e1007694 (2018).

Moneua, Y., Arenzana-Seisdedos, F. & Lortat-Jacob, H. The sweet spot: how GAGs help chemokines guide migrating cells. J. Leucoc. Biol. 99, 935–953 (2016).

Freeman, M. R., Delrow, J., Kim, J., Johnson, E. & Doe, C. Q. Unravelling glial biology: Gen target genes regulating glial development, diversification, and function. Neuron 38, 567–580 (2003).

MacDonald, J. M. et al. The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons. Neuron 50, 869–881 (2006).

Musashie, T. D., Purice, M. D., Spese, S. D., Doherty, J. & Logan, M. A. Insulin-like signaling promotes glial phagocytic clearance of degenerating axons through modulation of Draper. Cell Rep. 16, 1838–1850 (2016).

Kurashii, T. et al. Pretaporter, a Drosophila protein serving as a ligand for Draper in the phagocytosis of apoptotic cells. EMBO J. 28, 3868–3878 (2009).

Lin, L. et al. Complement-related regulates autophagy in neighboring cells. Cell 170, 18–171 e158 (2017).

McLaughlin, C. N., Perry-Richardson, J. J., Coutinho-Budd, J. C. & Broihier, H. T. Dying neurons utilize innate immune signaling to prime glia for phagocytosis during development. Dev. Cell 48, 506–522 e506 (2019).

Doherty, J. et al. PI3K signaling and Stat92E converge to modulate glial responsiveness to axonal injury. PLoS Biol. 12, e1001985 (2014).

Bach, E. A. et al. GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo. Gene Expr. Patterns 7, 323–331 (2007).

Doherty, J. & Logan, M. A., Tasdemir, O. E. & Freeman, M. R. Ensheathing glial function as phagocytes in the adult Drosophila brain. J. Neurosci. 29, 4768–4781 (2009).

Sepp, K. J., Schulte, J. & Auld, V. J. Developmental dynamics of peripheral glia in Drosophila melanogaster. Glia 30, 122–133 (2000).

Lewis, E. B. & Barker, F. Method of feeding ethyl methane sulfonate (EMS) to Drosophila males. Dros. Inf. Serv. 43, 193 (1968).

Hiller, K., Grote, A., Scheer, M., Munch, R. & Jahn, D. PrediSi: prediction of signal peptides and their cleavage positions. Nucleic Acids Res. 32, W375–379 (2004).

Ling, B., Larsson, B., von Heine, G. & Sonnhammer, E. L. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567–580 (2001).

References
1. Luo, L. & O’Leary, D. D. Axon retraction and degeneration in development and disease. Annu. Rev. Neurosci. 28, 127–156 (2005).

2. Neuson, L. J. & Freeman, M. R. Diverse cellular and molecular modes of axon degeneration. Trends Cell. Biol. 24, 515–523 (2014).

3. Schuldiner, O. & Yaron, A. Mechanisms of developmental neurite pruning. Cell. Mol. Life Sci. 72, 101–119 (2015).

4. Neniskyte, U. & Gross, C. T. Errant gardeners: glial-cell-dependent synaptic pruning and neurodevelopmental disorders. Nat. Rev. Neurosci. 18, 658–670 (2017).

5. Watts, R. J., Hooper, E. D. & Luo, L. Axon pruning during Drosophila morphogenesis: evidence for local degeneration and requirement of the ubiquitin–proteasome system. Neuron 38, 871–883 (2003).

6. Yu, F. & Schuldiner, O. Axon and dendrite pruning in Drosophila. Curr. Opt. Neurobiol. 27, 192–198 (2014).

7. Boulanger, A. & Dura, J. M. Nuclear receptors and Drosophila neuronal remodeling. Biochim. Biophys. Acta 1849, 187–195 (2015).

8. Yaniv, S. P. & Schuldiner, O. A fly’s view of neuronal remodeling. Wiley Interdiscip. Rev. Dev. Biol. 5, 618–635 (2016).

9. Awasaki, T. & Ito, K. Engulfing action of glial cells is required for programmed axon pruning during Drosophila morphogenesis. Curr. Biol. 14, 668–677 (2004).

10. Watts, R. J., Schuldiner, O., Perrino, J., Larsen, C. & Luo, L. Glia engulf degenerating axons during developmental axon pruning. Curr. Biol. 14, 678–684 (2004).

11. Tasdemir-Yilmaz, O. E. & Freeman, M. R. Astrocytes engage unique molecular programs to engulf pruned neuronal debris from distinct subsets of neurons. Genes Dev. 28, 20–33 (2014).
Acknowledgements

We thank Amélie Babled, Pascal Carme, and Dana Bis-Brewer for help in the EMS mutagenesis, MB developmental studies, and WGS analysis, respectively, and Oren Schuldiner for discussions about the Orion expression and function. We also thank Marc Freeman for alrnn-Gal4 stock, Baeg Gyong Hun for 10X-STAT92E-GFP stock, the Bloomington Drosophila Stock Center and VDRC for flp stocks, the BioCampus RAM-Drosophila facility (Montpellier, France), the imaging facility MRI, which is part of the UMS BioCampus Montpellier and a member of the National Infrastructure France-Bioimaging, supported by the French National Research Agency (ANR-10-INBS-04) for help in confocal and image analysis and processing. We acknowledge BDGP, BestGene, GenScript, and Genewiz for cDNA clone, transgene service, gene synthesis, and DNA-sequencing, respectively. The 1D4 anti-Fasciclin II hybridoma and the 8D12 anti-Repo monoclonal antibody developed by Corey Goodman and the 8A1 anti-Draper monoclonal antibody developed by Mary Logan were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. C.T. was supported by grants from the INSBB at the CNRS and from the Fondation pour la Recherche Médicale. Work in the laboratory of J.-M.D. was supported by the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer (grants SFI20121205950 and PIA 20151203422) and the Fondation pour la Recherche Médicale (Program “EQUIPES FRM2016” project DEQ20160334870).

Author contributions

A.B. and J.-M.D. designed the project; A.B., C.T., and J.-M.D. performed the experiments; A.B., S.Z., L.G.F., H.L.-J., and J.-M.D. analyzed the data; A.B. and J.-M.D. wrote the original draft of the manuscript; A.B., L.G.F., H.L.-J., and J.-M.D. reviewed and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22054-x.

Correspondence and requests for materials should be addressed to A.B. or J.-M.D.

Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021