

Neuronal programmed cell death induces glial cell division in the adult *Drosophila* brain

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Although mechanisms that lead to programmed cell death (PCD) in neurons have been analysed extensively, little is known about how surrounding cells coordinate with it. Here we show that neuronal PCD in the *Drosophila* brain induces glial cell division. We identified PCD in neurons and cell division in glia occurring in a consistent spatiotemporal manner in adult flies shortly after eclosion. Glial division was suppressed when neuronal PCD was inhibited by ectopic expression of the caspase inhibitor gene *p35*, indicating their causal relationship. Glia also responded to neural injury in a similar manner: both stab injury and degeneration of sensory axons in the brain caused by antennal ablation induced glial division. Eiger, a tumour necrosis factor superfamily ligand, appears to be a link between developmental PCD/neural injury and glial division, as glial division was attenuated in *eiger* mutant flies. Whereas PCD soon after eclosion occurred in *eiger* mutants as in the wild type, we observed excess neuronal PCD 2 days later, suggesting a protective function for Eiger or the resulting glial division against the endogenous PCD. In older flies, between 6 and 50 days after adult eclosion, glial division was scarcely observed in the intact brain. Moreover, 8 days after adult eclosion, glial cells no longer responded to brain injury. These results suggest that the life of an adult fly can be divided into two phases: the first week, as a critical period for neuronal cell death-associated glial division, and the remainder.

KEY WORDS: Neuronal programmed cell death, Glial division, *Drosophila*, Adult brain, Injury, Critical period

INTRODUCTION

Neurons are often made in excess during development. In vertebrates, many neurons that fail to connect to their targets are eliminated shortly after birth by caspase-dependent programmed cell death (PCD). Neuronal PCD therefore seems indispensable for creating appropriate relationships between neurons so as to form functional neural networks (Oppenheim, 1991). Despite intensive studies of the underlying mechanisms of such PCD (Brade, 1989; Clarke, 1985; Frebel and Wiese, 2006), little is known about how surrounding cells respond to the loss of their neighbours.

Developmental elimination of subcomponents of neurons is known to be associated with the activity of the surrounding cells. Axons, dendrites and synapses are also often created in excess, and unnecessary parts of them are eliminated in the course of neural circuit refinement in later stages. In the neuromuscular junctions, reduction of synapses and axon branches after birth is achieved in cooperation with the surrounding Schwann cells (Bishop et al., 2004). In the brain of *Drosophila melanogaster*, specific parts of the axon branches are pruned during metamorphosis by the engulfing activity of the surrounding glial cells (Awasaki and Ito, 2004; Watts et al., 2004). Adult neural circuits are not formed properly if this glial activity is blocked (Awasaki et al., 2006).

Glial cells are also involved in the response to accidental neural cell death. Astrocytes, Schwann cells and oligodendrocyte precursors proliferate after diverse types of neural injury, ranging from spinal cord injury to ischemia (Fawcett and Asher, 1999). Cell debris or factors released from damaged cells, such as nucleosides, are suggested to be involved in triggering the glial response. Moreover, glial proliferation is influenced by growth factors and cytokines such as tumour necrosis factor α (TNF α) and interleukin 6 (IL6) (Fields and Burnstock, 2006; Liu et al., 1995).

It is known that, in some cases, caspase-dependent cell death induces proliferation of the surrounding cells. In *Drosophila*, X-ray irradiation of the imaginal wing discs causes PCD and leads to compensatory cell proliferation (Haynie and Bryant, 1977). Expression of the pro-apoptotic gene *head involution defective* (*hid*; *Wrinkled* – FlyBase) or *reaper* (*rpr*) activates PCD through the apical caspase DRONC (*Drosophila* Nedd2-like caspase). In imaginal discs, DRONC also induces expression of *wingless* and *decapentaplegic* (*dpp*), and this promotes proliferation of the surrounding cells. In this case, therefore, the proliferation is triggered by cell death signalling rather than by the cell death itself. Both X-ray irradiation and the expression of *hid* or *rpr* cause overproliferation even when the cell death is inhibited by the caspase inhibitor *p35* (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004).

The programmed elimination of neural processes and accidental death of neurons are closely associated with the glial activity, but it was not known whether developmental cell death of neurons could also be associated with glial responses such as proliferation. Because of the wide variety of genetic manipulation techniques available, *Drosophila melanogaster* provides a good model system to address this issue. In the ventral nerve cord soon after adult fly eclosion, neurons that strongly express the Ecdysone receptor type A isoform (EcRA) respond to ecdysone signals by expressing *rpr* and *grim*, but not *hid*, and undergo PCD (Robinow et al., 1993). Ecdysone signalling is also known to directly induce *dronc* expression during larval PCD (Cakouros et

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al., 2004). It is not known, however, whether similar PCD also occurs in the brain and whether glial cells show any responses to such neuronal PCD.

Here we report that neuronal PCD also occurs in the *Drosophila* brain after adult eclosion. We show that glial cell division is induced by the PCD. Unlike in the wing disc, glial division after neuronal PCD is more likely to be triggered by molecular mechanisms similar to those involved in injury responses. The tumour necrosis factor Eiger is involved in glial division induced by both developmental PCD and injury. Secondary PCD is observed in the absence of Eiger and hence in the absence of glial division. In addition, we report the existence of a critical period of glial division, which is limited to just the first week of a fly's adult life, which can be as long as 50 days.

MATERIALS AND METHODS

Stocks and preparation

We used *Canton-S* (CS) strains as the wild-type control. We confirmed that *Canton-S* and *white¹¹⁸* strains showed essentially the same average number of BrdU-positive cells around the antennal nerve. *elav-p35* (Booth et al., 2000), *elav-GAL4* and *UAS-p35* strains were gifts from A. Hidalgo. *eiger¹* and *eiger³* (Igaki et al., 2002) were gifts from M. Miura. *UAS-eiger* (Moreno et al., 2002) was a gift from K. Basler. *GAL4-NP577* was used as a glial *GAL4* expression driver. For mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999), flies with the following genotypes were generated: *hs-FLP, tubP-GAL80, FRT 19A/FRT19A; UAS-lacZ/Actin-GAL4*, and *hs-FLP, tubP-GAL80, FRT 19A/FRT19A; UAS-GFP T2/Actin-GAL4*. All observations were made in female flies.

To minimise the effect of variation in the nutritional conditions among the flies, which could affect the growth rate of the animals, the number of flies per vial was strictly controlled. Thirty larvae were collected over a period of 6 hours after hatching and kept in a vial at 25°C until eclosion. Adult flies were collected within 6 hours after eclosion, and ten female and ten male flies were raised in a vial.

Definition of the areas of investigation

For the quantitative comparison of the labelled cells in the area around the root of the antennal nerve, we defined the region that includes the antennal nerve and the two-cell-thick layer of the surrounding cortex. Along the longitudinal axis of the antennal nerve root, we analysed the area between the entrance point of the antennal nerve and the level at which the dorsal population of the neural cell bodies around the antennal nerve disappears. Statistical analysis was performed using Excel (Microsoft) with statistics add-in software (Esumi, Tokyo, Japan).

Immunohistochemistry

Flies were anaesthetised with carbon dioxide and brains dissected in PBS and fixed with 4% formaldehyde in PEM (100 mM PIPES, 2 mM EGTA, 1 mM MgSO₄, pH 6.95) for 50 minutes at room temperature (RT). Incubations with primary and fluorescent-conjugated secondary antibodies were performed at 4°C overnight. To detect BrdU, the brains were treated with 2 M HCl for 20 minutes at RT after immunolabelling for other proteins. Nuclei were stained either with propidium iodide (PI) (Wako Pure Chemical industries, Osaka, Japan; 2 µg/ml in PBT) or TOTO3 (Molecular Probes; 1:2000 in 50% glycerol in PBS) for 3 hours at RT. Samples were analysed by confocal microscopy (Carl Zeiss LSM510 or Leica TCS SP2). Three-dimensional reconstructed images were generated with Zeiss software and processed with Photoshop (Adobe, San Jose, CA).

Antibodies used in this study were: mouse anti-BrdU (Beckton Dickinson, Franklin Lakes, NJ; 1:250; or GE Healthcare, Amersham, UK; 1:100), rat anti-ELAV (Developmental Studies Hybridoma Bank; 1:250), rabbit anti-REPO (gift from G. Technau; 1:250), mouse anti-EcRA (Developmental Studies Hybridoma Bank; 1:1000), rabbit anti-GFP (Molecular Probes, Eugene, OR; 1:1000), rabbit anti-β-galactosidase (ICN Pharmaceuticals, Aurora, OH; 1:4000), rabbit anti-FITC (Molecular Probes; 1:2000), rabbit anti-cleaved caspase 3 (Cell Signaling Technology, Beverly, MA; 1:100) and Alexa Fluor 488, 568 and 647-conjugated secondary antibodies (Molecular Probes; 1:250).

In situ DNA 3'-end labelling

For TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) analysis, free 3'-OH ends of DNA in the specimen were labelled with FITC using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland). In some cases, signals were enhanced by immunostaining with anti-FITC antibody.

BrdU incorporation

To label DNA-replicating cells, staged pupae were injected with ~0.1 µl of 3.5 mg/ml BrdU (Sigma, St Louis, MO) in PBS, and staged adult flies were fed yeast food containing 3.5 mg/ml BrdU. For longer treatment, flies were transferred to a new vial containing fresh food with BrdU every day. For pulse-chase labelling experiments, flies were fed food containing BrdU and red dye for 2 hours, and then transferred to normal food without BrdU. Half of the flies were fixed after 6 hours, and the remainder after 5 days. The red colour in the intestine of the flies disappeared quickly after they were transferred to the normal food. Therefore, it is unlikely that BrdU remains in the fly body for long after the pulse-labelling period to influence the number of BrdU-positive cells recorded in flies that were fixed later. In fact, the proportion of antennal nerves with BrdU-positive cells was almost the same in both conditions (28%, 17 of 60 samples after 6 hours; and 32%, 21 of 66 samples, after 5 days).

MARCM analysis

The flies for MARCM analysis (see above) were raised at 19°C to avoid spontaneous activation of *hs-FLP*. One day after eclosion, the flies were heat shocked for 1 hour at 37°C and maintained for 3 hours at 19°C. This cycle was repeated four times during the same day. Flies were raised for a further 3 days at 19°C. For the experiment combining BrdU labelling and MARCM analysis, flies were fed BrdU from the time of eclosion.

In situ mRNA hybridisation

In situ hybridisation was performed essentially as described previously (Ito et al., 2003), except that the specimens were treated with 0.3% H₂O₂ in methanol for 20 minutes at RT before they were subjected to in situ hybridisation. DNA probes used were *reaper*, *hid* and *grim*, which were synthesised using cDNAs as template (details available upon request).

Microsurgery

Prior to brain injury, flies were fed BrdU for 1 day. They were anaesthetised with carbon dioxide and a needle was inserted into the dorsal right-hand area of the head. The flies were then fed BrdU for another day before dissection and fixation. To induce degeneration of the antennal nerve axons, an antenna of the right side of the head was removed from the first segment, and the flies were then fed BrdU for a few days before dissection and fixation.

RESULTS

Neuronal programmed cell death in the adult fly brain

We first explored whether PCD occurs in the adult brain. At 6 hours after adult eclosion (AAE), anti-cleaved caspase 3 antibodies, which detect the active *Drosophila* caspases ICE and DCP1 (Yu et al., 2002), labelled cells in various regions of the brain (Fig. 1A). The labelling was observed most prominently and consistently in the area around the root of the antennal nerve (Fig. 1A, arrowheads). We therefore focused on this area for further analysis. The majority of the active caspase-positive cells were also labelled by TUNEL (10 out of 11 cells) and showed condensed nuclei (Fig. 1B), confirming that they underwent PCD. Such cells were only found during the first 2 days of adult life (Fig. 1C).

Immunostaining with a neural marker, ELAV, and a glial marker, REPO, revealed that the cells that undergo PCD are neurons (TUNEL-positive, ELAV-positive and REPO-negative) (Fig. 1D). Immunolabelling of caspase revealed that at least some of the dying neurons project their neurites into the antennal nerve (Fig. 1E). In the adult ventral nerve cord, some neurons that strongly express EcRA express the PCD-associated genes *rpr* and

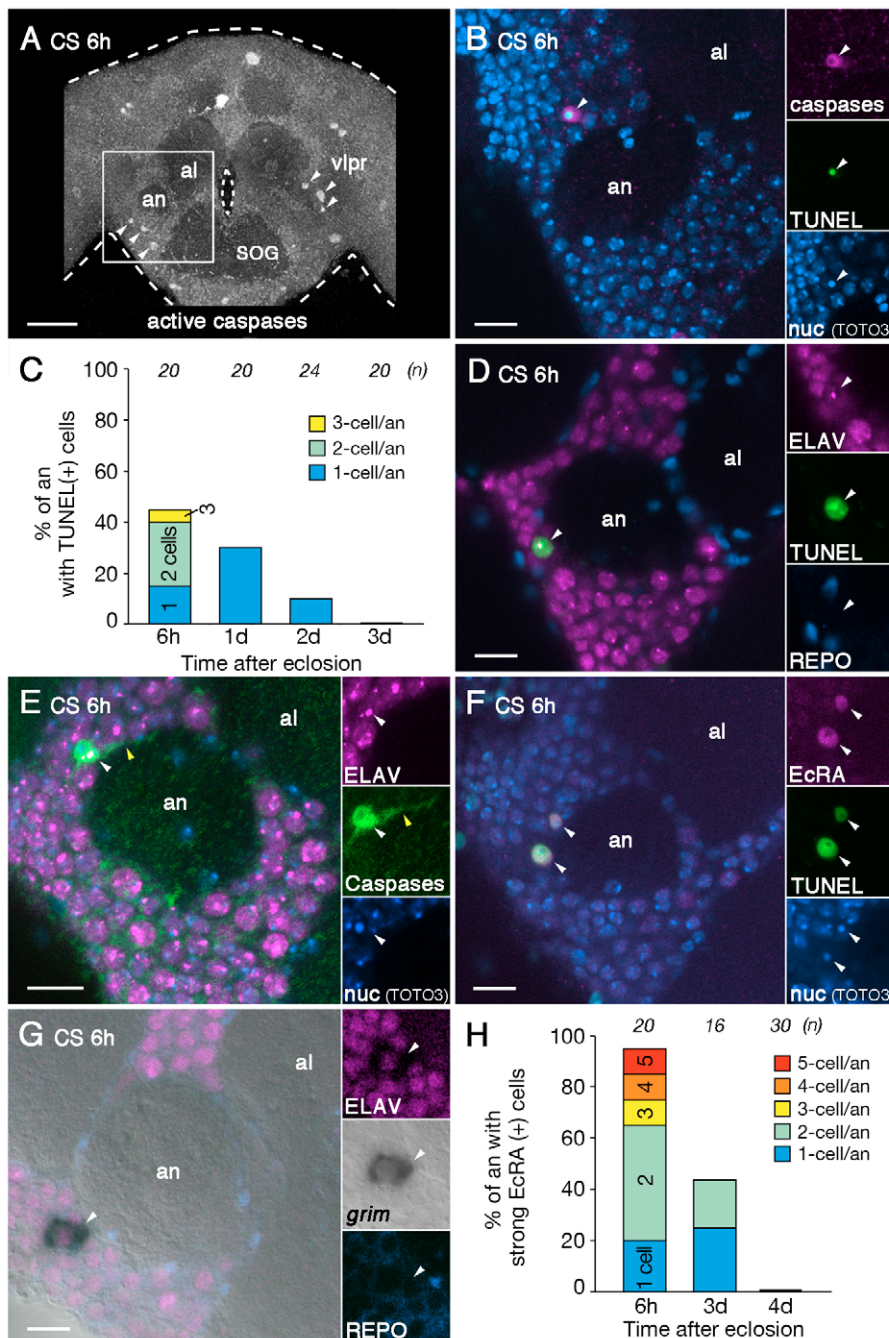


Fig. 1. Programmed cell death around the antennal nerve after adult eclosion. All images show frontal views of the fly brain [wild-type *Canton-S* (CS) after 6 hours of eclosion] with dorsal up. **(A)** Cells labelled with anti-cleaved caspase 3 antibodies are observed in various brain regions, including the area around the antennal nerve (an, arrowheads). Projection of confocal optical sections of the frontal half of the brain. Dashed lines outline the brain. The rectangle indicates the area shown in B,D-G. **(B,D-G)** A merged image (left) and single-channel images (insets) around the area of the labelled cells (arrowheads). A caspase-positive cell (B, arrowhead) was also TUNEL-positive and had a condensed nucleus. A TUNEL-positive cell (D, arrowhead) expressed the neural marker ELAV, but not the glial marker REPO. A caspase-positive ELAV-positive neuron (E, white arrowhead) sends its neurite towards the antennal nerve (yellow arrowhead). Projection of ten confocal sections. The cells that strongly expressed EcRA were also TUNEL-positive (F, arrowheads). A *grim*-expressing cell (G, whole-mount in situ hybridisation of *grim* mRNA) expressed the neural marker ELAV, but not the glial marker REPO (arrowhead). **(C,H)** Time course of the percentage of the antennal nerves with TUNEL-positive (C) and strongly EcRA-positive (H) cells. Colour coding of each bar indicates the percentage of antennal nerves with the indicated numbers of positive cells. The sample number (*n*) is indicated above each bar. an, cross-section of the root of the antennal nerve; al, antennal lobe; SOG, subesophageal ganglion; vlpr, ventrolateral protocerebrum. Scale bars: 50 μ m in A; 10 μ m in B,D-G.

grim, but not *hid*, and undergo PCD (Robinow et al., 1997; Robinow et al., 1993). Similarly, we found that strongly EcRA-positive neurons around the antennal nerves underwent PCD (20 out of 43 strongly EcRA-positive cells were also TUNEL-positive) (Fig. 1F). Consistently, we found neurons that expressed *grim* (75% of the antennal nerves observed, $n=8$) (Fig. 1G) and *rpr* (67%, $n=6$), but not *hid* (0%, $n=20$) (data not shown). Compared with TUNEL-positive cells, strongly EcRA-positive cells were observed more frequently and for a longer period, of up to 3 days AAE, which is 1 day longer than for TUNEL-positive cells (Fig. 1H). This might be because some strongly EcRA-positive neurons escape cell death. In addition, the opportunity to detect TUNEL-positive cells is relatively restricted: TUNEL

detects only the short final phase of PCD, whereas the dying cells appeared to express EcRA for a longer period, starting before the execution of PCD.

The spatiotemporal pattern of cell division coincides with PCD

We then asked whether caspase-dependent PCD in the adult brain would induce cell division, as observed in the imaginal wing discs (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). We analysed the distribution of DNA-replicating cells by bromodeoxyuridine (BrdU) incorporation. When flies were fed BrdU for a 1-day period from the mid-pupal stage to 10 days AAE, BrdU-positive cells were found most prominently in the

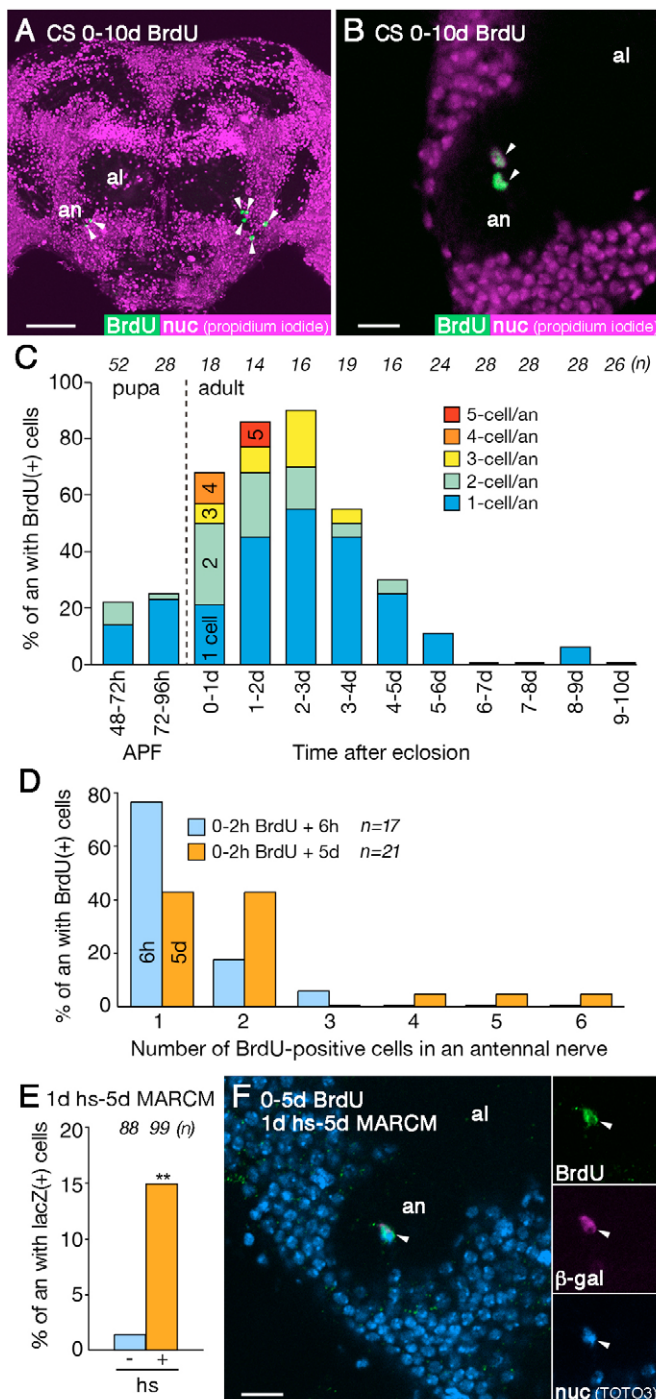


Fig. 2. Glial division around the antennal nerve after eclosion.

(A,B) An adult fly brain of the wild-type (CS) strain after 10 days of BrdU treatment. Projection of confocal optical sections of the frontal half of the brain (A) and high-magnification single section of the right hemisphere around the antennal nerve (B). BrdU-positive cells were found predominantly around the antennal nerve (arrowheads). (C) Percentage of the antennal nerves associated with BrdU-positive cells. The colour coding indicates the percentage of antennal nerves with the indicated numbers of labelled cells. The sample number (n) is indicated above each bar. (D) Pulse-chase labelling experiment. Blue and orange bars show the percentage of antennal nerves with the indicated numbers of BrdU-positive cells. (E) Percentage of antennal nerves with *lacZ*-positive cells induced by MARCM. -, flies without heat shock; +, flies with heat shock to induce MARCM clones. **, $P < 0.01$ (Fisher's exact test). (F) A MARCM-labelled cell (expressing β -galactosidase, arrowhead) also incorporated BrdU. Flies were heat shocked 1 day after eclosion and fixed 4 days later. Frontal optical section around an antennal nerve. A merged image (left) and single-channel images (insets) are shown. Scale bars: 50 μ m in A; 10 μ m in B,F.

cell division would make it difficult to detect M phase, the number of BrdU-positive cells being, on average, 2.3 for a 10-day labelling. We therefore employed two alternative approaches – a pulse-chase experiment and MARCM labelling – that do not detect a specific phase of the cell cycle but reflect the progression of cell division.

For the BrdU pulse-chase labelling, newly eclosed flies were fed BrdU for 2 hours and fixed after either 6 hours or 5 days (Fig. 2D). If the cells that incorporated BrdU underwent mitosis, the number of labelled cells should increase. Indeed, antennal nerves with more than three BrdU-positive cells were found only in the flies fixed after 5 days, whereas after 6 hours most of the antennal nerves had only a single labelled cell (76%, 13 of 17 cells). Consistently, the number of labelled cells increased significantly after 5 days (average 2.0 versus 1.3 cells, $P < 0.05$, Mann-Whitney's U-test), whereas the frequency of antennal nerves with BrdU-positive cells was almost the same in both conditions (see Materials and methods).

In the MARCM technique (Lee and Luo, 1999), reporter gene expression is induced via mitotic recombination caused by expression of heat shock-activated *Flippase*. Cells are therefore labelled only when there is mitosis. In the heat-shocked flies, antennal nerves with *lacZ*-expressing cells were observed much more often than without heat shock (Fig. 2E) ($P < 0.01$, Fisher's exact test). Furthermore, cells labelled by MARCM coincided with the BrdU-labelled cells (Fig. 2F) (14 MARCM-positive cells among 28 BrdU-positive cells). These results demonstrated that cells around the antennal nerve do indeed divide.

Dividing cells in the adult brain are glial cells

To determine the identity of the dividing cells, we combined BrdU labelling with immunostaining for glial and neuronal markers. Immediately after a 3-hour BrdU labelling, almost all the BrdU-positive cells expressed the glial marker (REPO-positive and ELAV-negative; 98%, 47 of 48 cells) (Fig. 3A,B). Thus, most of the dividing cells are likely to be glia. After a 1-day BrdU labelling followed by a 9-day chase period, most BrdU-positive cells were also glia (REPO-positive and ELAV-negative; 88%, 37 of 42 cells) (see Fig. S1A in the supplementary material). MARCM analysis with a cytoplasmic GFP reporter combined with 5-day BrdU

area around the antennal nerve (Fig. 2A,B), which corresponds to the area where prominent PCD was observed. The number of BrdU-labelled cells in this area reached a maximum slightly after the peak of PCD, and BrdU-positive cells were detected for a few days after PCD had ceased (compare Fig. 2C with Fig. 1C,H). This spatiotemporal coincidence might imply the induction of cell division in response to PCD (discussed below).

We then tried to label the dividing cells with antibody to the M-phase marker phosphorylated histone H3 (PH3), considering that BrdU incorporation does not necessarily mean cell division: DNA might be synthesised without mitosis (endoreplication). We could not, however, detect any positive signals. The low frequency of

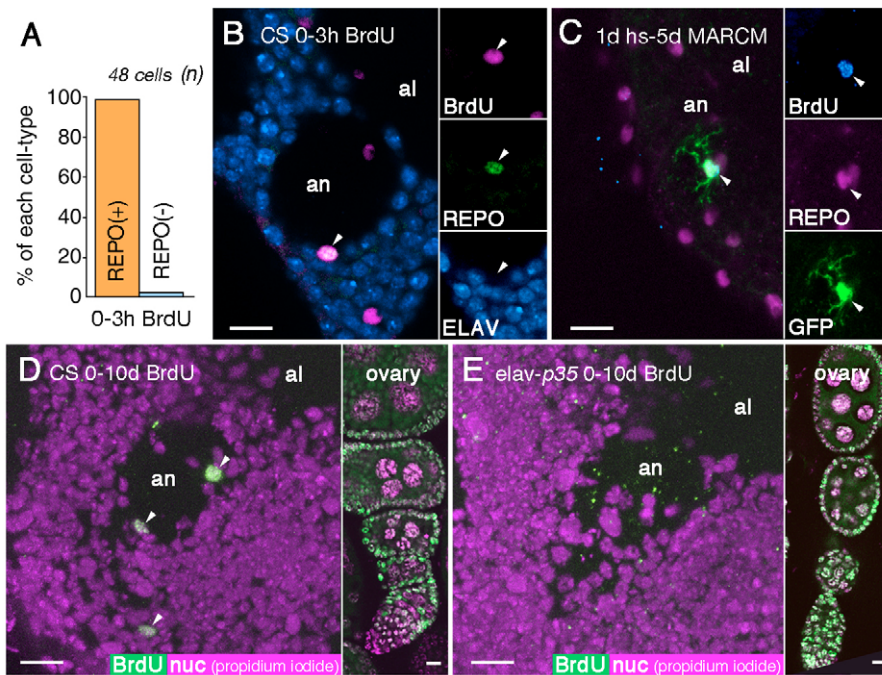


Fig. 3. Glial cell division induced by neuronal PCD. (A) The percentage of cells that are REPO-positive or -negative among the BrdU-positive cells observed around the antennal nerve after a 3-hour BrdU treatment (0-3h BrdU). The sample number (n) is indicated above the bar. (B,C) Frontal optical section around an antennal nerve. Dorsal is up. A merged image (left) and single channel images (insets) are shown. (B) A BrdU-positive cell expressed the glial marker REPO, but not the neural marker ELAV (arrowhead). (C) Morphology of a BrdU-positive REPO-positive glial cell visualised by MARCM (expressing GFP, arrowhead). Flies were heat shocked 1 day after eclosion and fixed 4 days later. (D,E) Specimens after 10 days of BrdU treatment. Projection of confocal optical sections around the antennal nerves (left), and a single optical section of the ovary (right). (D) Wild-type (CS) flies. BrdU-positive cells were observed both in the brain (arrowheads) and ovary. (E) The *elav-p35* strain, in which neuronal PCD was inhibited by continuous ectopic expression of p35 in neurons. BrdU incorporation in the brain was suppressed specifically. Scale bars: 10 μ m.

treatment revealed that BrdU-positive REPO-positive cells extended multiple processes of irregular shape in various directions, showing typical glial structure (Fig. 3C).

There was a small population of REPO-negative ELAV-negative BrdU-positive cells (3-hour BrdU labelling: 2%, 1 of 48 cells; 1-day BrdU labelling: 2%, 1 of 42 cells) (Fig. 3A and see Fig. S1 in the supplementary material). Because of the sporadic and low frequency occurrence of this cell type, we were not able to visualise the morphology of these cells with MARCM. Although their exact identity therefore remains unresolved, our results nevertheless suggest that the vast majority of the dividing cells are REPO-positive glial cells.

Glial cells are classified according to differences in their position and morphology, which are implicative of functional differences (Ito et al., 1995). Interestingly, the BrdU-positive glial cells were located within or very close to the neuropiles, but not deep in the cortex or along the brain surface (Fig. 2B, Fig. 3B). It is therefore likely that only a distinct subset of glia responds to PCD.

Neuronal PCD induces glial cell division

The spatiotemporal coincidence between neuronal PCD and glial cell division suggests that the former might induce the latter. To examine this causal relationship, we utilised a fly strain, *elav-p35* (Booth et al., 2000), in which execution of neuronal PCD is inhibited by a virus-derived caspase inhibitor, p35. If cell death signalling induces cell division, as observed in the imaginal wing discs, this would be expected to increase the number of BrdU-positive cells. After 10 days of BrdU feeding, however, labelled cells were observed only around the antennal nerves of wild-type (90%, 9 of 10) (Fig. 3D, left), but not of *elav-p35* (0%, 0 of 22) (Fig. 3E, left) flies, even though both strains showed successful BrdU incorporation in the ovary, where egg cells divide actively in the adult (Fig. 3D,E, right). The same results were obtained when expression of p35 was driven by *elav-GAL4* ($n=20$). The fact that p35 inhibits glial cell division in the adult brain suggests that it is features of the dead or dying neurons (e.g. membrane degeneration), rather than the signalling pathways associated with the PCD, that trigger glial division.

Brain injury and axonal degeneration induce glial cell division

If dead or dying neurons trigger glial division, one might ask whether there is any similarity with the response to neural lesion, which is known in adult animals ranging from cockroaches to vertebrates (Fawcett and Asher, 1999; Treherne et al., 1984). *Drosophila* glia are able to proliferate upon genetically induced neural ablation during embryogenesis (Griffiths and Hidalgo, 2004), and retinal degeneration mutants (*rdgB^{KS222}*) are characterised by excessive glia in the degenerated adult tissue (Stark and Carlson, 1982). We therefore asked how glia would respond to adult brain injury.

To address this, we performed antennal ablation (Fig. 4A), in which sensory nerves from the antenna undergo degeneration (Stocker et al., 1990). To avoid endogenous glial division induced by the neuronal PCD, we ablated the antenna of the *elav-p35* flies. When BrdU was administered from 0 to 3 days AAE, BrdU-positive glial cells were observed only around those antennal nerves that had antennal ablation (Fig. 4B,C, first column). Thus, it is likely that ablation-induced degeneration of axons is sufficient to cause the glial proliferation response.

When the antennae of wild-type flies were ablated, coexistence of endogenous PCD and ectopic axonal degeneration was expected in these flies. However, the number of BrdU-positive cells did not increase prominently ($P>0.05$, Fisher's exact test) (Fig. 4C, second column). Although injury might activate more glial cells and/or trigger another round of cell division, for the most part, the same glial cells are likely to be activated by PCD and axonal degeneration.

We then examined whether glial cells in other parts of the brain have the ability to respond to injury. Using a needle, we stabbed the dorsal area of the right-hand head capsule of flies fed with BrdU (Fig. 4D). One day after the stabbing, we found ectopic BrdU-positive glial cells around the injury site, but not in the intact hemisphere (Fig. 4E,F), suggesting that glia that do not normally divide retain the ability to react upon injury. Although we could not exclude the possibility that endoreplication might occur in some cells, our data, as well as those of a previous report (Stark and Carlson, 1982), suggest that adult brain injury induces glial cell division.

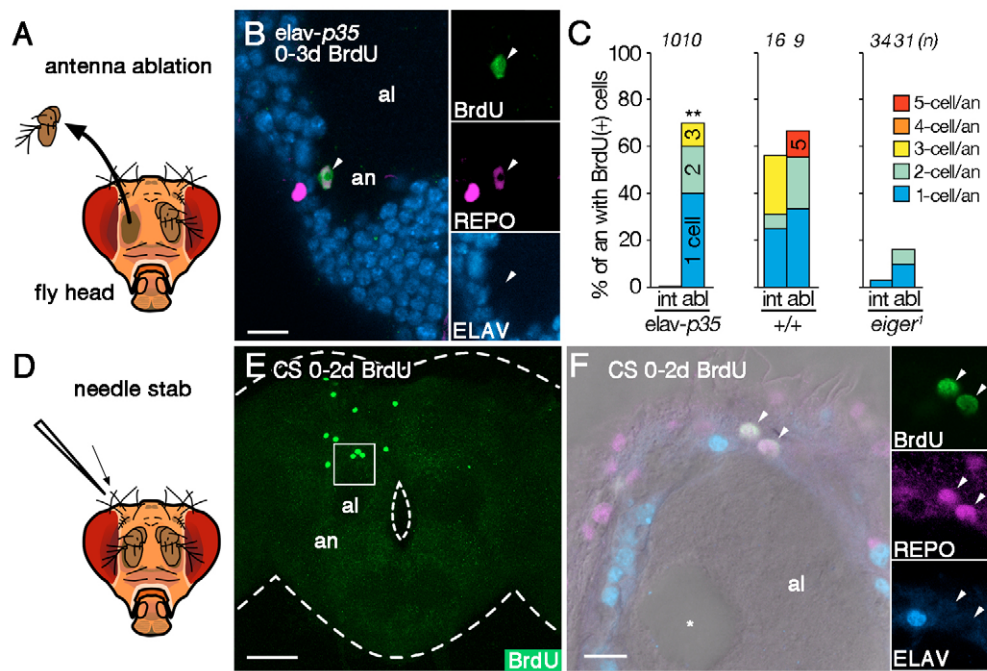


Fig. 4. Neural injury-associated glial cell division. (A) Schematic of the antennal ablation. An antenna on the right side was ablated from the first antennal segment. (B) Antennae were ablated soon after eclosion, and the flies were fed BrdU for 3 days. Antennal ablation induced BrdU incorporation into glial cells (REPO-positive, arrowhead) even though neuronal PCD was inhibited by p35. (C) Percentage of the antennal nerves with BrdU-positive cells. Colour coding indicates the percentage of antennal nerves with the indicated numbers of labelled cells. int, antennal nerves with intact antennae; abl, antennal nerves with antennae ablated just after eclosion. *elav-p35*, 3-day BrdU treatment; +/+, wild type (CS), 5-day BrdU treatment; *eiger¹*, homozygous *eiger* mutant, 5-day BrdU treatment. **, $P < 0.01$ (Fisher's exact test). The sample number (n) is indicated above each bar. (D) Schematic of the needle stab. After feeding the flies with BrdU for 1 day, a needle was inserted into the brain, and the flies were fed BrdU for another day before fixation. (E) Projection of confocal optical sections of the frontal half of the brain. Dashed lines outline the brain. BrdU-positive cells were found around the region of injury. (F) An optical section of the area indicated in E (boxed). A montage of confocal bright-field and immunostaining images. BrdU-positive cells were REPO-positive but ELAV-negative, showing that they were glia (arrowheads). Asterisk indicates the site of injury. Scale bars: 50 μm in E; 10 μm in B,F.

Eiger is a common link between PCD/neural injury and glial cell division

As the same glial cells are likely to respond to both PCD and neural injury around the antennal nerves, we asked whether the same signalling molecule mediates these responses. TNF α , a TNF superfamily ligand, is tightly involved in the response to neural lesion in mammals (Scherbel et al., 1999). We therefore explored whether a known *Drosophila* TNF superfamily ligand, Eiger (Igaki et al., 2002; Moreno et al., 2002), is involved in either or both responses.

In homozygous *eiger* mutants, the frequency of PCD was essentially the same as that of wild-type flies during the first 2 days of adult life (Fig. 5A). In spite of this, essentially no BrdU incorporation was observed in homozygous mutant flies of two independent *eiger* alleles (*eiger¹* and *eiger³*) after 5 days of BrdU treatment (Fig. 5B, first, sixth and seventh columns) ($P < 0.01$, Bonferroni test after Fisher's exact test extended to $r \times c$). This suggests that Eiger plays a role in mediating signals between neuronal PCD and glial cell division. Expression of *eiger* using the glial driver GAL4-NP577 (see Fig. S2 in the supplementary material) in a homozygous *eiger* mutant background increased the number of BrdU-positive cells significantly (Bonferroni test, $P < 0.05$) (Fig. 5B, fifth and sixth columns). This confirms that the function of Eiger is necessary for the glial response. However, the rescue was not complete, suggesting that spatiotemporal expression of *eiger* is required. In addition, ectopic expression of *eiger* alone

did not cause overproliferation of glial cells (Bonferroni test, $P > 0.05$) (Fig. 5B, third and fourth columns), suggesting the involvement of other molecules.

Interestingly, the loss of Eiger function caused increased neuronal PCD at a later period. At 3 days AAE, PCD occurred considerably more frequently in *eiger* mutants than in the wild type ($P < 0.05$, Fisher's exact test) (Fig. 5A). This suggests that either Eiger or glial division has a protective function against PCD.

We then tested whether Eiger also mediates glial responses against neural lesion. Needle stab into *eiger* mutant brains appeared to induce BrdU incorporation much less frequently than in the wild type (data not shown). To examine the effect of injury quantitatively, we measured the frequency of BrdU incorporation after antennal ablation. Injury only occasionally induced BrdU incorporation in *eiger* mutants (Fig. 4C). This suggests that Eiger is also involved in mediating the glial response to neural lesion. The fact that *eiger* mutation attenuates both types of glial division, as induced by PCD and non-PCD events (antennal ablation), suggests that the same signalling molecule, Eiger, mediates these responses.

A critical period of glial cell division in response to PCD and neural injury

Do glial cells retain their mitotic ability to respond to neural loss during aging? The number of cells in the adult brain decreases significantly between 6 and 30 days AAE (T. Shimada, M. Kamiya, K.K. and K.I., unpublished observation). We therefore investigated

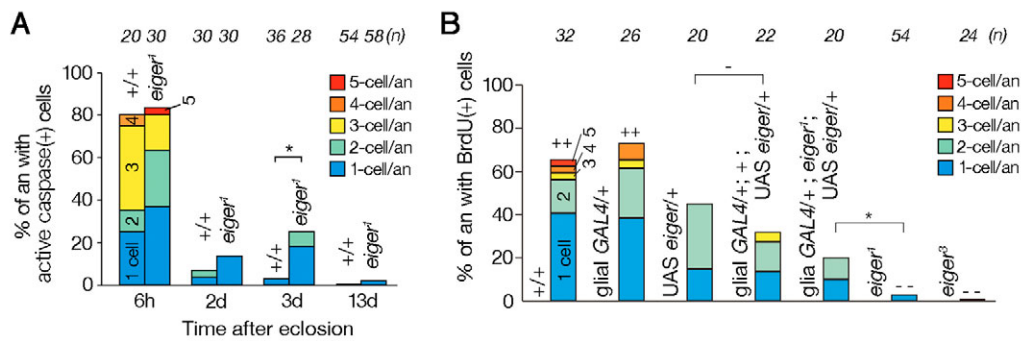


Fig. 5. Involvement of Eiger in glial division. (A) Time course of the percentage of antennal nerves with active caspase-positive cells. *, $P < 0.05$ (Fisher's exact test). (B) Percentage of antennal nerves with BrdU-positive cells after 5-day BrdU treatment. +/+, wild type (CS); *eiger¹* and *eiger³*, homozygous *eiger* mutants; *glial GAL4*, *GAL4-NP577*. Genotypes that are responsible for significant differences are marked by ++ and -- above the bars, indicating high and low levels of BrdU incorporation, respectively ($P < 0.01$, adjusted residual test after Fisher's exact test extended to $r \times c$ performed for the seven genotypes analysed). *, $P < 0.05$ and --, $P > 0.05$ (Bonferroni test). The number of BrdU-incorporating cells in the flies carrying *UAS-eiger* alone was slightly lower than in the wild type, possibly because of the difference in genetic background. Colour coding shows the percentage of antennal nerves with the indicated numbers of labelled cells. The sample number (n) is indicated above each bar.

BrdU incorporation in the brain for each 10-day period until 50 days AAE, which effectively covers most of the lifetime of *Drosophila* in laboratory conditions. Consistent with the results described above, BrdU incorporation was observed in the flies that were fed BrdU for the first 10 days of adult life (Fig. 6A). None of the samples showed BrdU-positive cells after 10 days AAE (Fig. 6A). This indicates (1) that glial cells divide only during a specific period early in adult life, and (2) that the dividing glial cells are not a significant contributor to cell number in the adult fly brain.

We examined whether glia retain the potential to divide after PCD-associated glial division has ceased (6 days AAE) (Fig. 2C) by injuring the brains of older flies by antennal ablation. BrdU incorporation was observed only up to 8 days AAE and not thereafter (Fig. 6B). Even severe damage, such as stab injury, did not cause glial division in older flies (Fig. 6C). Altogether, glial division in response to neural loss seems to be a unique feature of the first week of adult life.

In addition to the fact that we did not find any BrdU-positive cells after 10 days AAE, it is important to note that we did not find any BrdU-positive ELAV-positive cells (i.e. neurons) anywhere in the brain, i.e. neither around the antennal nerve nor in any other areas.

Thus, unlike in vertebrates and some insects (Cayre et al., 1996; Garcia-Verdugo et al., 2002), neurons do not newly arise in the *Drosophila* adult brain throughout its lifetime.

DISCUSSION

Developmental PCD of neurons induces glial division in the *Drosophila* adult brain

In this study, we found that neurons in specific areas of the *Drosophila* brain undergo PCD over several days AAE. Similar to the PCD in the ventral nerve cord, dying neurons strongly express *EcRA* and also express the pro-apoptotic genes *rpr* and *grim*, but not *hid*. These findings suggest that the same ecdysone-mediated developmental mechanism is utilised for eliminating unnecessary neurons in the brain and the ventral nerve cord.

We proved our hypothesis that neuronal PCD induces glial cell division. Most of the cells that incorporated BrdU were glia. We found a very small number of BrdU-positive cells that were both REPO-negative and ELAV-negative. A conceivable candidate is neural stem cells (neuroblasts). However, this is unlikely because BrdU-positive neurons have never been observed. The identity of this novel cell type remains to be investigated.

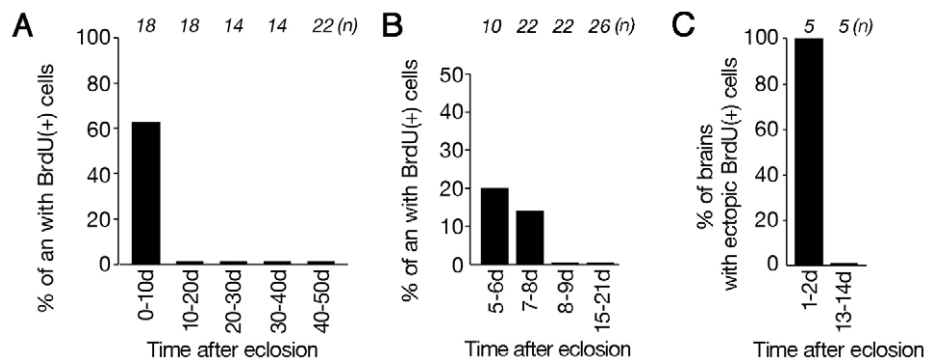


Fig. 6. Competence of glial division during adult life. (A) Time course of the percentage of antennal nerves with BrdU-positive cells after a 10-day-long feeding of BrdU until 50 days after adult eclosion. (B) Time course of the percentage of antennal nerves with BrdU-positive cells after antennal ablation. Antennae were ablated at the beginning of each period shown on the x-axis, and the flies were fed BrdU until the end of the period indicated. (C) Percentage of the brain with ectopic BrdU-positive cells around the site of injury. A needle was inserted on the day shown on the x-axis. The sample number (n) is indicated above each bar.

That neuronal PCD occurs in essentially all individuals was indicated by the fact that strongly EcRA-positive cells were observed in most flies at 6 hours AAE (Fig. 1H). In spite of this, not all the antennal nerves were found associated with BrdU-positive cells (Fig. 6A). This discrepancy might be due to the technical difficulty of labelling cells with BrdU for long periods: some of the BrdU-incorporating cells might have died as BrdU is potentially toxic.

In the imaginal wing disc, preventing cell death itself with p35, but leaving the caspase signalling pathway intact, increases proliferation (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). This was not the case for glial division in the brain because glial cells did not divide when cell death was suppressed by p35. Apparently, a different molecular mechanism triggers the glial response. We found that Eiger, a TNF superfamily ligand, is involved in this process. Glial division in both intact and injured brains was attenuated in *eiger* mutants, and ectopic expression of *eiger* in glia rescued this phenotype. The rescue, however, was not complete, and glial expression of *eiger* alone did not induce ectopic glial division. This might be because (1) spatiotemporal expression of *eiger* is required in glia, (2) expression of *eiger* in the neurons might also be important, or (3) factors other than Eiger are also involved in this process.

What, then, could be the role of glial division upon developmental PCD? In the *Drosophila* *rdgB*^{KS222} mutant, glial cells in the compound eye fill the voids that were formed by axonal degeneration (Stark and Carlson, 1982). Similarly, dividing glial cells in the brain might contribute to structural support after neural loss. Another possibility is that glial cells protect neural tissue by removing dead cells and/or by secreting trophic factors. Our observation that the lack of Eiger, and thus the lack of glial division, led to the increase in neuronal PCD supports this hypothesis.

Neural injury induces glial division in the *Drosophila* adult brain

The *Drosophila* adult brain shows a similar injury response to that of vertebrates: expression of β amyloid protein precursor-like (APPL) and activation of c-Jun N-terminal kinases (JNKs) are induced (Leyssen et al., 2005). Neurons fail to regenerate in response to injury (Ayaz et al., 2008), and glial cells in the antennal lobe change their morphology upon antennal ablation (Macdonald et al., 2006). Glial division, by contrast, has not been demonstrated in the fly brain. Here, we provided evidence that glia also divide upon injury and that Eiger mediates this process.

The glial division observed in the fly brain, however, seems to be much less extensive than that observed in vertebrates. A notable difference is in the variety of the dividing glial cell types. Whereas astrocytes, microglia and oligodendrocyte precursor cells proliferate in vertebrates (Fawcett and Asher, 1999), only a subset of glial cells around the neuropile is likely to respond in the *Drosophila* brain. As drastic glial proliferation upon neural injury, which causes a glial scar, is involved in the inhibition of neural recovery in vertebrates (Yiu and He, 2006), the *Drosophila* nervous system, with its much restricted level of glial division, should provide an interesting model system for investigating the responses of neurons to injury, including neural recovery.

In vertebrates, TNF α is involved in the inflammatory response against neural lesions and plays multiple roles in such as the induction of cell death, cell survival and proliferation through the JNK and NF κ B pathways (Goetz et al., 2004; Varfolomeev and Ashkenazi, 2004; Scherbel et al., 1999). In *Drosophila*, overexpression of *eiger* in the imaginal discs appears to cause

caspase-dependent cell death through the JNK pathway via Wengen, the sole known TNF receptor (Kauppila et al., 2003; Moreno et al., 2002). However, Eiger is not required for caspase-dependent cell death caused by ionising radiation of the imaginal discs, even though irradiation induces the expression of *eiger* (Brodsky et al., 2004). Eiger is known to contribute in vivo to the proper localisation of determinant during the asymmetric division of neuroblasts (Wang et al., 2006). Wengen, however, does not seem to be involved in this process, suggesting the existence of as yet unknown receptors for Eiger. In our study, RNA interference of *wengen* did not appear to cause defects in glial division (data not shown). Further investigation is required to understand the pathways downstream of Eiger in glial cell division, as well as in various other Eiger-mediated phenomena.

A critical period of glial division upon neural loss

A surprising finding of our study is that there is a critical period of glial division. We found that both PCD- and injury-induced glial division only occur during the first 8 days AAE. Glial cells that are distant from the antennal nerve, which do not normally divide in the adult brain, retain the ability to respond to brain stab. This competence is lost as the flies grow older. Interestingly, there is temporal coincidence between the competence of glial division and neural plasticity. The application of certain odorants leads to an increase in the volume of particular glomeruli only during 2-5 days, but not after 8 days, AAE (Sachse et al., 2007; Devaud et al., 2003). Considering the possible role of glia in trophic function and structural support, glial division might be actively involved in brain plasticity. The temporal coincidence suggests that the adult stage of *Drosophila* can be divided into two phases: the first week AAE, as the critical period in which glial division against neural loss and plasticity of the antennal lobe neurons can be observed, and the rest of the adult life, during which these events do not occur.

Our study has identified the first example in which developmental PCD triggers glial cell division. We also revealed important similarities between the glial response to PCD and to neural injury and between the glial response in insects and vertebrates after injury. The model system introduced in this study serves as a convenient platform for analysing novel types of neuron-glia interaction during recovery of the brain after PCD and injury, as well as how stage-dependent glial competence is controlled.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/1/51/DC1>

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