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## REVIEW ESSAY

## Prospects &amp; Overviews

# Neuron-glia crosstalk in neuronal remodeling and degeneration: Neuronal signals inducing glial cell phagocytic transformation in *Drosophila*

Ana Boulanger | Jean-Maurice Dura 

IGH, Université de Montpellier, CNRS, Montpellier, France

**Correspondence**

Ana Boulanger and Jean-Maurice Dura, IGH, Centre National de la Recherche Scientifique, Université de Montpellier, Montpellier F-34396, France.  
Email: ana.boulanger@igh.cnrs.fr and jean-maurice.dura@igh.cnrs.fr

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**Abstract**

Neuronal remodeling is a conserved mechanism that eliminates unwanted neurites and can include the loss of cell bodies. In these processes, a key role for glial cells in events from synaptic pruning to neuron elimination has been clearly identified in the last decades. Signals sent from dying neurons or neurites to be removed are received by appropriate glial cells. After receiving these signals, glial cells infiltrate degenerating sites and then, engulf and clear neuronal debris through phagocytic mechanisms. There are few identified or proposed signals and receptors involved in neuron-glia crosstalk, which induces the transformation of glial cells to phagocytes during neuronal remodeling in *Drosophila*. Many of these signaling pathways are conserved in mammals. Here, we particularly emphasize the role of Orion, a recently identified neuronal CX<sub>3</sub>C chemokine-like secreted protein, which induces astrocyte infiltration and engulfment during mushroom body neuronal remodeling. Although, chemokine signaling was not described previously in insects we propose that chemokine-like involvement in neuron/glia cell interaction is an evolutionarily ancient mechanism.

**KEYWORDS**

brain development, *Drosophila* neurogenetics, glial cell phagocytic transformation, neuronal remodeling, neuronal signals, neuron-glia crosstalk

**INTRODUCTION**

Developmental neuronal remodeling encompasses removal or addition of neurites or synapses within already formed neuronal circuits at later developmental stages. Pruning is defined as the developmentally regulated degeneration of axons, dendrites, and synapses. Pruning involves both cell autonomous (i.e., within the neuronal cells) as well as non-cell autonomous (i.e., involving surrounding tissues) mechanisms. Neurite pruning is often followed by regrowth of neurites that are adapted

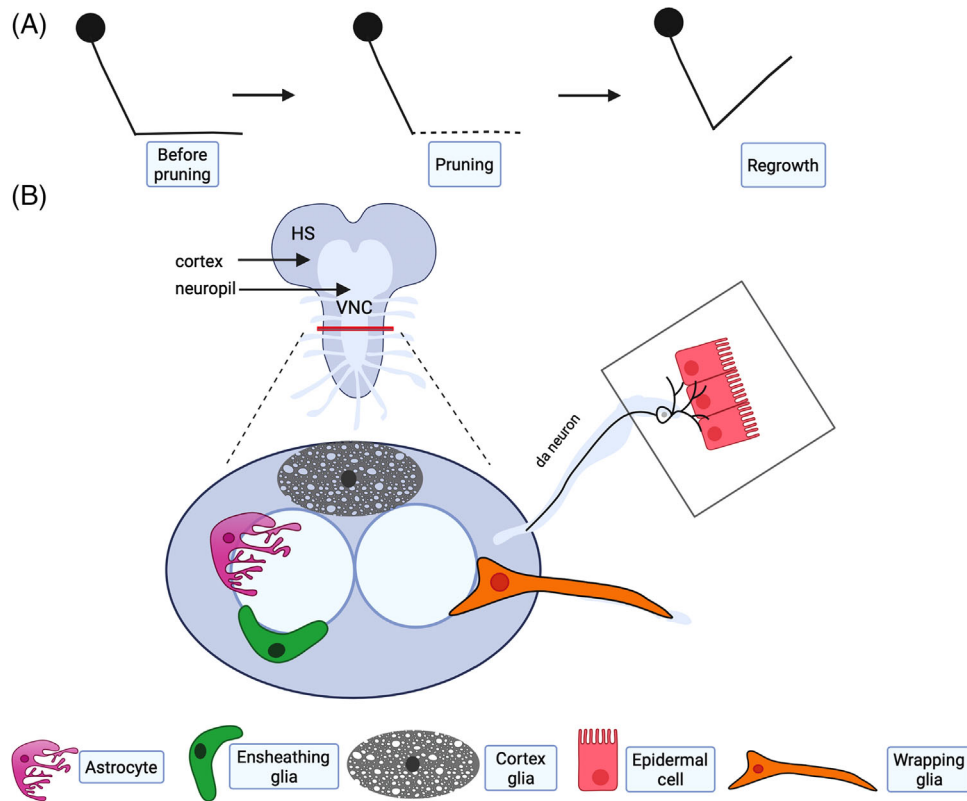
to the next developmental stage (Figure 1A). Cell autonomous mechanisms can involve gene expression changes<sup>[1,2]</sup> and local disassembly of the cytoskeleton.<sup>[3]</sup> Non-cell autonomous mechanisms prominently involve communication with surrounding tissues, in particular here glial and epidermal cells, and activation of their phagocytotic machinery. These non-cell autonomous mechanisms are the main focus of this review.

Physiological developmental neuronal remodeling can affect neurites only but can also result in the loss of the neuron cell body itself. This developmental remodeling is a widely used mechanism, across the animal kingdom, to refine neurite targeting necessary for the maturation of the neural circuits which in turn is necessary for their function.

**Abbreviations:** APF, after puparium formation; APP, amyloid precursor protein; CNS, central nervous system; da neuron, dendritic arborization neuron; ORN, olfactory receptor neuron; PNS, peripheral nervous system; PS, phosphatidylserine; VNC, ventral nerve cord

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**FIGURE 1** (A) General scheme of axonal pruning and regrowth during neuronal remodeling. Only a part of the axon is pruned and axon regrowth allows the neuron to connect different targets before and after pruning. (B) *Drosophila* glial and epidermal phagocytic cell types involved in developmental neuronal remodeling and injury. The larval *Drosophila* CNS is composed of two brain HS and the VNC. Nerves connect the VNC to the peripheral structures. The cortex houses most neuronal and glial cell bodies. CNS axonal contacts between neurons are found in the neuropil. The cross-section view shows the morphology and location of the four glial subtypes displaying phagocytic activity: astrocytes, ensheathing glia, cortex glia, and wrapping glia. The top right of the panel shows a row of epidermal cells connecting a sensory da neuron. CNS, central nervous system; HS, hemispheres; VNC, ventral nerve cord

Similar molecular and cellular mechanisms are at work during neurodevelopmental disorders or after nervous system injury.<sup>[4-9]</sup> In particular, the predominant phagocytes acting during the neuronal remodeling and degeneration are glial cells and critical signaling pathways between glia and neurons have been identified.<sup>[7-9]</sup> The elimination of neuronal debris by glial cells can be divided into three different cellular steps. The first step is infiltration of the glial cells. The second step is the recognition/engulfment of neuronal debris. The third step is the phagocytosis through the endocytosis of the engulfed debris by the glial cells resulting in the formation of phagosome which subsequently matures. Very little is known about the relationship between the signaling pathways activated in the glia and the onset of phagosome formation and further maturation.

Communication signals between neurons and glia to regulate development and injury are still poorly understood. In mammals, unwanted synapses are tagged by complement for microglia elimination and this process becomes aberrantly reactivated in neurodegenerative disease.<sup>[10-12]</sup> These glial responses, although important for minimizing neuronal damage, may also contribute to the progression of some neurodegenerative disorders, such as Alzheimer's and Huntington's diseases.<sup>[13]</sup>

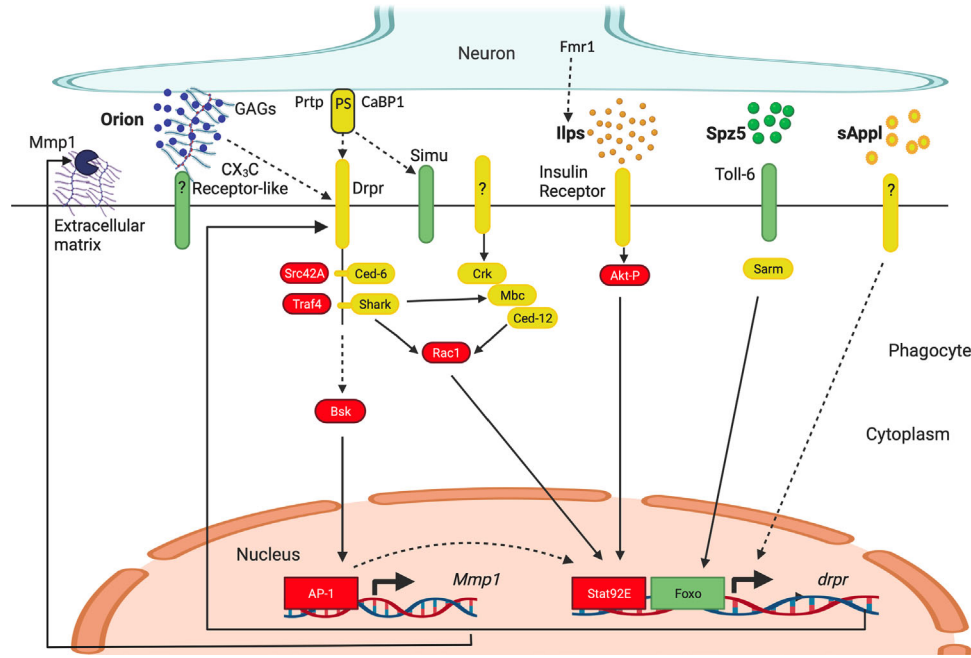
### *Drosophila* glial cell types

The *Drosophila* nervous system comprises seven morphologically defined glial cell types. Four of these glial cell types are found only in the central nervous system (CNS): astrocyte-like glia, ensheathing glia, midline glia, and cortex glia; one cell type, wrapping glia, is found only in the peripheral nervous system (PNS), and two cell types are found in both the CNS and the PNS (perineurial glia and subperineurial glia).<sup>[14]</sup> Among these seven glial cell types, phagocytic activity is proposed in four of them: astrocyte-like glia, ensheathing glia, cortex glia, and wrapping glia. They are located in different regions of the brains (Figure 1B): astrocytic-like glia cell bodies reside at the cortex/neuropil interface, but they extend major branched processes into the neuropil, ensheathing glia ensure compartmentalization between the neuropil and the cortex region but do not infiltrate the neuropil, cortex glia envelop all neuronal cell bodies in the cortex area of the CNS and wrapping glia wrap individual axons or axon bundles in the PNS. These glial cells are thought to be activated by different signals sent from neurons which, in turn, initiate axon bundle infiltration and/or engulfment of neuronal debris followed by their destruction in phagosomes. Glial activation requires close proximity between the neuron to be destroyed and the

**TABLE 1** Neuron-glia crosstalk during development and disease in *Drosophila*

Stage/context	System	Ligand	Phagocytic cell	Receptor	Effectors	References
Development (L2-early pupae)	Optic lobe apoptosis	Prtip, PS, CaBP1	Cortex glia	Drpr	Ced-6/Shark; Crk/Mbc/ced-12; Rac1	[49]
Development (L3)	Neuronal Apoptosis brain + VNC	Spz5	Cortex glia	Toll-6	Sarm/Foxo/ <i>drpr</i> transcription	[51]
Development (early pupae)	da neurons	PS	Epidermal epithelial cells	Drpr		[28,29]
Development (early pupae)	MB	Orion	Astrocytes	Unknown		[71]
Development (early pupae)	Corazonin neurons; MB		Astrocytes, others	Drpr + unknown	Crk/Mbc/Ced-12	[22]
Development (adult)	Pdf-Tri neurons	Ilps? activated by Fmr1?	Ensheating and cortex glia	InR	Drpr	[53]
Adult remodeling	Brain	sAppl	Glia	Unknown		[39]
Injury (larvae)	da neurons	PS	Epidermal epithelial cells	Drpr	Crq, Dsb	[28]
Injury	ORN		Ensheating glia	Drpr	Ced-6/Shark	[31]
Injury	ORN	Ilps	Ensheating glia	InR	Akt/Stat92E/ <i>drpr</i> transcription	[30]
Injury	ORN		Ensheating glia	Drpr + Unknown	Traf-4/ Src42A /Bsk/ Crk/Mbc/ced-12; Rac1/ Stat92E; <i>drpr</i> transcription	[32-34]
Injury	VNC (and ORN)		Ensheating glia	Drpr	AP-1/ <i>Mmp1</i> transcription; Stat92E	[40]
Injury + sleep	ORN		Glia	Drpr	Stat92E	[35]
Injury	Wing axons		Wrapping glia	Drpr		[42]
Injury	Wing axons (and bystander)		Glia	Drpr	Shark/Bsk/AP-1/ <i>Mmp1</i> transcription	[43]
Injury	ORN	sAppl	Glia	Unknown	Drpr	[39]
Pathological Htt aggregates	ORN (with or without injury)		Glia	Drpr	Shark/Bsk; Crk/Ced-12; Rac1	[44,45]
Pathological A $\beta$ peptides	Brain (antennal lobe)		Glia	Drpr	Stat92E; AP-1/ <i>Mmp1</i> transcription	[46]
Adult brain phagocytosis	<i>drpr</i> and <i>simu</i> overexpression	PS	Phagocytic glia	Drpr; Simu		[47]

da neuron, dendritic arborization neuron; MB, mushroom body; ORN, olfactory receptor neuron; PS, phosphatidyserine; VNC, ventral nerve cord. When not specified, injury is done at the adult stage.



**FIGURE 2** A proposed model of neuronal signals, receptors, and pathways involved in activation of phagocytic cells in *Drosophila*. Seven different ligands presented or secreted by neurons: Orion, Prtp, PS, CaBP1, Ilps, Spz5, and sAppl are thought to activate phagocytic pathways via a putative CX<sub>3</sub>CR-like, Drpr, Simu, InR, Toll-6 receptors, and an unknown receptor. Receptor activation by these ligands controls, in turn, a cascade of signaling pathways that at the end activates transcription factors, which positively regulate the expression of *Mmp1* and *drpr*. Increased production of Drpr is essential for glia infiltration, debris engulfment, and phagocytosis. Other proteins such as *Mmp1* are needed for the extracellular matrix degradation during remodeling of neural circuits in response to neural activity during development and brain damages. Where receptors ligands and effectors have been described in both developmental and injury pathways they are shown in yellow. Where they were described only in developmental pathways or injury-mediated pathways, they are shown in green and red, respectively. Dashed lines represent putative interactions or pathways

reacting glial cells. However, in some cases where glia phagocytes are not local or they cannot reach the site of clearance, neighboring cell types, for example, epidermal cells, can act as phagocytes playing significant roles in the phagocytosis and clearance of neuronal debris.<sup>[15,16]</sup>

## Signaling pathways

The draper (*drpr*) gene plays a central essential role in phagocytic activation in both glial and epidermal phagocytic cells (see references in Table 1). The *drpr* gene was first identified as a downstream target of *glial cells missing*. It encodes a transmembrane domain receptor, homologous to the *Caenorhabditis elegans* gene *ced-1* and the human *MEGF10* and *MEGF11* genes, which are expressed by glial cells and macrophages. Drpr mediates phagocyte recognition of dying cells required for cell corpse removal in the CNS.<sup>[17]</sup> Drpr is also involved in neuronal remodeling, where it is required for neurite pruning and/or cell body removal. Several proteins have been shown to mediate Drpr signaling: the adapter protein Ced-6, bearing a phosphotyrosine binding domain, Shark and Src42A, two nonreceptor tyrosine kinases, and the TNF receptor-associated factor 4 (Traf4). The four proteins bind to the intracellular region of Drpr. A first cascade of signaling leads to the activation of the Rac1 GTPase and this results in the phosphorylation of transcription

factors such as Stat92E that positively regulate the expression of *drpr*. Drpr is also involved in a second cascade of signaling, that involves the Bsk kinase (*basket*), which leads to the transcription of genes, such as *Mmp1* (*matrix metalloproteinase 1*), that are regulated by the AP1-complex (Figure 2). Several ligands have been proposed for the Drpr receptor. Prtp (*Pretaporter*) resides in the endoplasmic reticulum and is exposed at the cell surface after induction of apoptosis.<sup>[18]</sup> CaBP1, another endoplasmic reticulum protein, is required for apoptosis.<sup>[19]</sup> Prtp and CaBP1 are biochemically capable of binding Drpr, but binding in neurons and glia has not been shown. The *Drosophila* macroglobulin complement-related (Mcr) appeared to signal through Drpr to regulate autophagy.<sup>[20]</sup> Phosphatidylserine (PS) is a phospholipid present in the inner leaflet of the plasma membrane (facing cytoplasm) and is exposed to the extracellular milieu, after flipping to the outer leaflet in cells undergoing apoptosis. It has been proposed that apoptotic cells externalize PS as an “eat-me” signal for their phagocytic removal and that Drpr is a PS-binding receptor for phagocytosis.<sup>[21]</sup> A striking specificity of Drpr function was discovered during the remodeling of the larval ventral nerve cord (VNC) peptidergic vCrz<sup>+</sup> neurons. These neurons undergo apoptosis, and their neurites are eliminated during early metamorphosis. Drpr functions in nonastrocytic glia to clear vCrz<sup>+</sup> cell bodies but neurite debris clearance is largely accomplished by another astrocytic glia signaling pathway.<sup>[22]</sup>

Here, we review neuronal signals inducing glial activation during injury and pathological neurodegeneration as well as during developmental and adult neuronal remodeling. A particular attention is given to the recently identified CX<sub>3</sub>C chemokine-like Orion as a signal secreted by brain neurons, which activates glial cells and transforms them into phagocytes.

## SIGNALS IN INJURY-INDUCED NEURODEGENERATION

In mammals, two known pathways are at work to remove injured neurons: the complement pathway via the complement receptor CR3 and the fractalkine pathway via its microglial receptor CX<sub>3</sub>CR.<sup>[23,24]</sup> In mice, unilateral whisker removal is achieved by either whisker trimming or by whisker lesioning by cauterization. Whisker lesioning leads to synapse degeneration in the somatosensory cortex by sensory depletion. It was shown that the CX<sub>3</sub>CR, after being bound by cortical neuron-secreted chemokine, fractalkine, is responsible for the removal of neuronal debris following whisker lesioning. This neuron to microglia signaling depends on *Adam10*, a gene encoding a metalloprotease, which cleaves the fractalkine into a secreted form.<sup>[24]</sup>

In *Drosophila*, axotomy triggers reactions from local glia that are very similar to those observed in mammals. For example, severing adult olfactory nerves that project into the antennal lobes of the central brain initiates a classic Wallerian degeneration program in olfactory receptor neuron (ORN) axons.<sup>[25,26]</sup> Over the course of several days, local glia extends membranous projections into the antennal lobe neuropil to phagocytose degenerating axonal and synaptic debris. The neuronal signals and pathways that control glial migration to the sites of trauma and the phagocytic activity of glial cells are mostly unknown. A schematic representation of *Drosophila* injury assays was recently presented.<sup>[27]</sup>

In this section, we review neuron-glia crosstalk events during injury in larval neurons and adult ORNs, VNC, and wings. We will focus on what is known concerning the signals sent from injured neurons to glia or epidermal cells inducing phagocytic transformation.

### Dendritic arborization neurons signal epidermal epithelial cells via PS

The dendritic arborization neurons (da neurons) are a convenient system for studying injury-induced dendrite degeneration. da neurons are multidendritic sensory neurons growing underneath the larval body wall; their dendrites degenerate during metamorphosis. Laser severing of larval da neuronal dendrites triggers degeneration of distal dendrites and dendritic debris production. The primary cells responsible for the removal of the injury-resulting dendrite debris are adjacent epidermal cells. In these cells, *drpr* was found to be essential for the engulfment of the larval injury resulting debris. In addition, two members of the CD36 family of scavenger receptors, *Crq* (*croquemort*) and *Dsb* (*debris buster*), are needed for further phagosome maturation in

epidermal cells during da neuron debris clearance after injury. Thus, it was shown that debris persisted after larval injury in a *drpr* or *crq* mutant as compared to control.<sup>[28]</sup> Interestingly, even though the Drpr ligand in this system is still unknown, it was found that PS was specifically exposed on injured degenerating dendrites to function as an “eat-me” signal.<sup>[29]</sup> Consequently, after injury, exposed-PS debris might be recognized by Drpr on the epidermal cells, and thus be subsequently engulfed.

### Olfactory receptor neurons signal ensheating glia via Ilps and sAppl

*Drosophila* has two pairs of olfactory organs, the antennae and the maxillary palps, where the ORNs encode the variety of olfactory stimulus received. ORN cell bodies are housed in the third antennal segments or maxillary palps and the corresponding axons project to the antennal lobe of the brain via the antennal or maxillary nerves, respectively. This olfactory neuronal network is a commonly used system for the study of injury-induced activation of glia resulting from the simple mechanical removal of adult fly palps or antenna. This ablation completely removes ORN cell bodies and transects the nerve. Degeneration of severed ORN axons and glial responses to this injury are easily monitored in vivo for several weeks after injury.

Potential ligands proposed as mediators of the neuron-glia crosstalk after maxillary palp or antennal ORN axotomy are the Insulin-like peptides (Ilps) that belong to a class of injury-released factors. Ilps are packaged into dense core vesicles (DCV) for release from injured axons shortly after injury.<sup>[30]</sup> Ilps bind to the InR (*Insulin receptor*) in ensheating glia and this in turn activates glial phagocytic activity via Drpr to promote clearance of degenerating axons.

Initial studies showed that ensheating glia enwrap major structures in the adult brain as the antennal lobe and respond morphologically to axon injury. Thus, ensheating glia cells work as phagocytes in the adult brain in contrast to astrocytes, which do not respond, either morphologically or molecularly, to axotomy in this system.<sup>[31]</sup> Ensheating glia autonomously require not only *drpr* but also other key components of the glial phagocytic machinery such as *ced-6* and the nonreceptor tyrosine kinase *Shark* for successful clearance of degenerating axons from the injured brain. However, the Drpr ligand in this system has not yet been identified. Furthermore, it was shown that in addition to this Drpr-Ced-6-Shark pathway, a second separable nonredundant signaling pathway, for which the ligand and its membrane receptor are still unknown, is at work to modulate glial clearance of axonal debris after axotomy: one mediated by the guanine nucleotide exchange factor complex Crk/Mbc/Ced-12 (Figure 2). The Drpr pathway appears to act during early stages of glia activation by promoting extension of glial membranes to the degenerating axons. In contrast, the guanine nucleotide exchange factor pathway acts at later stages to promote phagocytosis of axon debris.<sup>[32]</sup> Both pathways converge on the modulation of the GTPase Rac1 to ensure the cytoskeletal remodeling essential for glial infiltration of injury sites and removal of damaged neurons.<sup>[33]</sup> The unknown injury signals received by Drpr at

the membrane are important regulators of downstream transcriptional responses in reactive glia. These responses are mediated by the transcription factors Stat92E and AP-1 complex which directly and indirectly increased *drpr* expression, respectively (Figure 2).<sup>[34]</sup>

Interestingly, physiologic processes such as sleep also promote the engulfment and accelerated clearance of damaged neurons. Recent studies have shown that drug-induced sleep induction accelerates the engulfment and clearance of damaged ORN neurites by glial membranes via the activation of *drpr* expression and Stat92E.<sup>[35]</sup> Conversely, in mechanically (shaking stimulus) and genetically (*quiver* (*qvr*)/*sleepless* mutants) sleep-deprived animals, multiple markers of glial activation were delayed which was associated with impaired clearance of damaged neurites.

The amyloid precursor protein (APP) is linked to the Alzheimer's disease as being the source of the amyloid  $\beta$  ( $A\beta$ ) peptide considered to be pathologic in the disease. APPs have been therefore investigated intensely; however, their normal function in the brain remains unclear and controversial. APP is a functionally and structurally conserved transmembrane protein, present in both vertebrates and invertebrates. APP can be cleaved by cellular secretases, releasing secreted APP (sAPP). *Appl* encodes a single APP homologue in *Drosophila* and is expressed in all neurons throughout development.<sup>[36–38]</sup> It was recently shown that *Appl* null individuals have defective clearance of degenerative axons, compared to controls, after antennal ablation. This mutant phenotype is rescued by full-length *Appl* as well as by sAppl. Moreover, loss of *Appl* causes reduced *Drpr* expression compared to control after ablation in antennal lobe glia. This indicates the possibility of a requirement for sAppl communication between the neuron to glia for clearance of axonal debris after injury.<sup>[39]</sup>

### Ventral nerve cord neurons signal ensheating glia through unknown factors

A novel model of injury developed in the VNC based on axotomy of nerve axon projections to the VNC from legs, wings, or head allowed the identification of the factor, *Mmp1* whose expression is *Drpr*-dependent and requires the Stat92E and AP-1 transcription factors (Figure 2).<sup>[40]</sup> Interestingly, the authors showed that this cascade of events was also conserved in injured ORNs. *Mmp1* expression is induced in ensheating glia response to axons severing and allows glia to properly infiltrate neuropil regions after axotomy and consequently to clear axonal debris. As during ORN injury, ensheating glia, but not astrocytes, responded to degenerating axons in the VNC by upregulating *Drpr*.

Neuron glia communication is also essential after nerve cord injury to regenerate neurons and glia that have been destroyed. These neurogenic responses require activation of the insulin pathway in glia. Thus, autocrine binding of the *Ilp6* ligand to the *InR* in cortex glia induces glial proliferation. In addition, binding of the *Ilp6* ligand to the *InR* in Nerve/Glial Antigen 2-like neuropile glia reprograms glia into neural stem cells for neuron and glia cell regeneration, thus restoring glial cell populations and priming for neurogenesis.<sup>[41]</sup>

### Wing nerve neurons signal glia through unknown factors

The *Drosophila* wing is an interesting system for injury studies since it allows for visualization of single axons by analysis of the nerve housed in the marginal (L1) wing vein, which contains both sensory neurons and glia. The cell bodies of these neurons are aligned along the length of the L1 vein and project their axons within the L1 vein into the thorax. Some of these axons project through the entire wing and are among the longest axons in *Drosophila*. Thus, axotomies can easily be performed through the middle of the L1 vein. Here also, unknown injury signals spread from the wing vein severed axons to glial cells, which engulf axonal debris. In the wing nerve, there are two main glial subtypes: wrapping glia that ensheath axons directly, and subperineurial glia that surround both axons and wrapping glia. By removing *drpr* in either type of glia, it was shown that wrapping glia are the key glial cell type required for clearance of axonal debris induced after axotomy in the L1 wing vein although a modest effect was also observed after the removal of *drpr* in the subperineurial glia.<sup>[42]</sup>

Degeneration of injured axons occurs in two different phases: a first phase of axon inactivation that occurs 1–3 h after injury, in which axonal transport is altered and a second phase of severed axon fragmentation that occurs between 6 and 12 h after injury. The  $NAD^+$  hydrolase *Sarm* plays a key function in the two phases and seems to act in different biochemical pathways at each phase. Interestingly, early injured axons are also responsible for the altered axon transport of uninjured adjacent neurons, named here bystander neurons. This happens in two steps: a first step in which inactive severed axons signal to glia resulting in the activation of *Drpr*, *Mmp1*, and other proteins, and a second step in which glia signal to bystander neurons and alter their physiology. The possibility that glia might be directly injured by the axotomy and signal to bystander neurons without input from the severed axons seems less probable.<sup>[43]</sup> Ligands allowing the communication between injured neurons and glial *Drpr* as well as between glia and bystander neurons have not yet been described in the wing injury system.

## SIGNALS IN NEURODEGENERATION MODELS

*Drosophila* models have also been used to investigate the role of phagocytic glia in clearance of pathogenic fragments of both *Htt* (*huntingtin*) associated with Huntington's disease and *Appl*. In this section, we summarize some data concerning the neuron-glia cross talk in *Drosophila* models of degenerative diseases and during genetic induction of brain degeneration.

### Huntington's disease model

Expansions of an N-terminal glutamine-rich polyQ tract in *Htt* above Q37 are pathogenic and result in insoluble aggregates that form cytoplasmic inclusions. In contrast, *Htt* fragments containing wild-type

polyQ ( $Q < 37$ ) are soluble but can be recruited onto “seeds” consisting of preformed  $Q > 37$  Htt aggregates. Htt fragments with polyQ  $> 37$  can induce aggregate formation in neurons and aggregate evolution can be followed thanks to fusion to fluorescent proteins. It was observed that the amount of Htt aggregates in ORN expressing neurons remains constant through adulthood, suggesting a balance of aggregate formation and degradation. To investigate the mechanism by which these aggregates were cleared, antenna axotomy was performed and it was observed that glia cells regulate Htt aggregate clearance in neurons through a mechanism that requires Drpr and its downstream phagocytic engulfment machinery.<sup>[44]</sup> Interestingly, it was shown that this Drpr-dependent aggregate clearance can also occur in the absence of injury. Based on these data, glia could be considered as protective against neuronal toxic aggregates. Interestingly, the authors also showed that after expression of both mutant Htt in neurons and wild-type Htt in glia, wild-type Htt colocalizes with mutant Htt in glia and aggregates, in a prion-like conversion effect, suggesting that glia may also spread Htt aggregates throughout the brain and contribute to their increased toxicity.<sup>[44]</sup>

Further studies have confirmed the pathogenic role of glia and have shown that Htt aggregates can travel between connected neurons in the adult fly olfactory system through the glial cytoplasm (neuron1  $\rightarrow$  glia  $\rightarrow$  neuron2), and this aggregate transfer also requires Drpr.<sup>[45]</sup> However, why aggregates sometimes escape the glial phagocytic machinery or how aggregates that invade the glial cytoplasm can transfer to neurons and spread as neuronal aggregates remain unsolved.<sup>[45]</sup> Interestingly, these data suggested that glia selectively target presynaptic neurons by recognizing an apoptotic “eat-me” signal induced by Htt aggregate accumulation. Based on these studies, one could suggest that Htt aggregates might be considered as a source of a neuronal signal activating phagocytosis in glia.

### Alzheimer’s disease model

Glial cells also offer protection against Alzheimer’s disease by engulfing extracellular  $A\beta$  peptide.<sup>[46]</sup> It is not clear if these peptides are directly signaling through Drpr, nevertheless, after neuronal expression of  $A\beta$ , Drpr and its downstream targets are activated. Moreover, neurodegeneration phenotypes observed after neuronal expression of  $A\beta$  in adult flies are more severe in *drpr* mutant animals. The authors favor the model that  $A\beta$  peptides released from neurons are engulfed by glia in a Drpr-dependent manner and travel through protein destruction pathways to promote their disintegration. Thus, degeneration in *drpr* mutants is most probably induced by toxicity due to the accumulation of unengulfed  $A\beta$  peptides. Another possibility could be that the absence of *drpr* leads to neurodegeneration due to a failure of overall glial support mechanisms. Recent studies have shown that in adult brains, overexpression of *drpr* or the glial transmembrane phagocytic receptor *Nimrod C4/six-microns-under (simu)*,<sup>[47,48]</sup> the *Drosophila* ortholog of Stabilin-2, increased phagocytic activity of glia promoting neurodegeneration. This reduction in neuronal number is not linked to neuronal apoptosis but rather to PS-mediated phagocytosis, since

neuronal loss can be rescued by masking PS with the milk fat globule-EGF factor 8 (MFG-E8).<sup>[47]</sup> Taken together, these data suggest that activated glia have a dual role, one that is protective, another toxic. Increased expression of phagocytic receptors could be beneficial to remove altered neurons but regulation of this increase is essential. Thus, the gain-of-function of glial phagocytic receptors potentially resulting from their abnormal high expression must be considered a risk factor in neurodegenerative disorders.

### SIGNALS FROM DEGENERATION OF HEALTHY NEURONS DURING DEVELOPMENTAL AND ADULT NEURONAL REMODELING

*Drosophila* is rich in neuronal remodeling paradigms during development, which can continue even after adult hatching. In this section, we review neuron-glia cross talk events during normal remodeling in healthy neurons. We discuss mechanisms occurring in healthy neurons independently from the events that occur post-trauma even though they can appear similar or even identical. It is presently thought that some differences may exist in the molecular nature of the neurite pruning in healthy neurons versus injury-induced neurite degeneration<sup>[25]</sup> (see also discussion in ref.[22]). The particular case of mushroom body neurite pruning during metamorphosis is reviewed in the last section.

#### da neurons signal epidermal cells via PS

Epidermal cells are the primary phagocytes of da neuron degenerating dendrites during developmental pruning from 5 h after puparium formation (APF) to 18 h APF. As for postinjury, Drpr, Crq, and Dsb are required for developmental debris clearance.<sup>[28]</sup> A direct causal relationship has been established between PS exposure and dendrite degeneration of the da neurons. How the Drpr receptor expressed by the phagocytes senses PS exposure is still poorly understood.<sup>[29]</sup>

#### Do larval optic lobe neurons use a combination of ligands to signal cortex glia?

Considerable amounts of neuronal cell death occur in the developing larval optic lobes through apoptosis. Subsequently, cell corpses are efficiently removed at the early pupal stage. The clearance of dead neurons is carried out by cortex glia via *drpr*-mediation mechanisms; *drpr* is expressed from the second instar to early pupal stages.<sup>[49]</sup> Using a strategy of RNAi-mediated gene knock-down in the glial cells, it was shown that *Shark* has a significant role in the clearance of dead neurons although only moderate roles were observed for *ced-6*, *Crk*, *mbc*, *Ced-12*, and *Rac1*. The authors suggest the possibility that Prtp, CaBP1, and PS function as Drpr ligands in this system although it was not shown that they are, in fact, expressed by neurons. However, Prtp and CaBP1 were not found to be essential for debris clearance.<sup>[49]</sup>



## Larval brain neurons signal cortex glia through Spz5

In the normal *Drosophila* L3 brain about 400 apoptotic debris, likely corresponding to dying neurons, can be visualized by cleaved Death caspase-1 (Dcp-1). This number nearly doubles in brain mutants for the Toll-6 receptor, which was first described for its role during embryonic development and later in the innate immune signaling.<sup>[50]</sup> This effect is attributed to the impaired Toll-6 expression in the cortex glia which surround the neuronal cell bodies. Neuronal apoptosis induces a Furin-mediated cleavage of the cytokine neurotrophin Spz5 whose secretion drives the activation of Toll-6 signaling in the cortex glia. The Toll-6 signaling, via the Sarm and Foxo pathway, activates *drpr* transcription which results in increased expression of Drpr receptor (Figure 2). The authors propose that Spz5 secreted from dying neurons has the characteristics of a “find-me” signal through the glial Toll-6 receptor different from the “eat-me” signal recognized by Drpr.<sup>[51]</sup>

## Do Pdf-Tri neurons signal glia through Fmr1 regulated Ilps release?

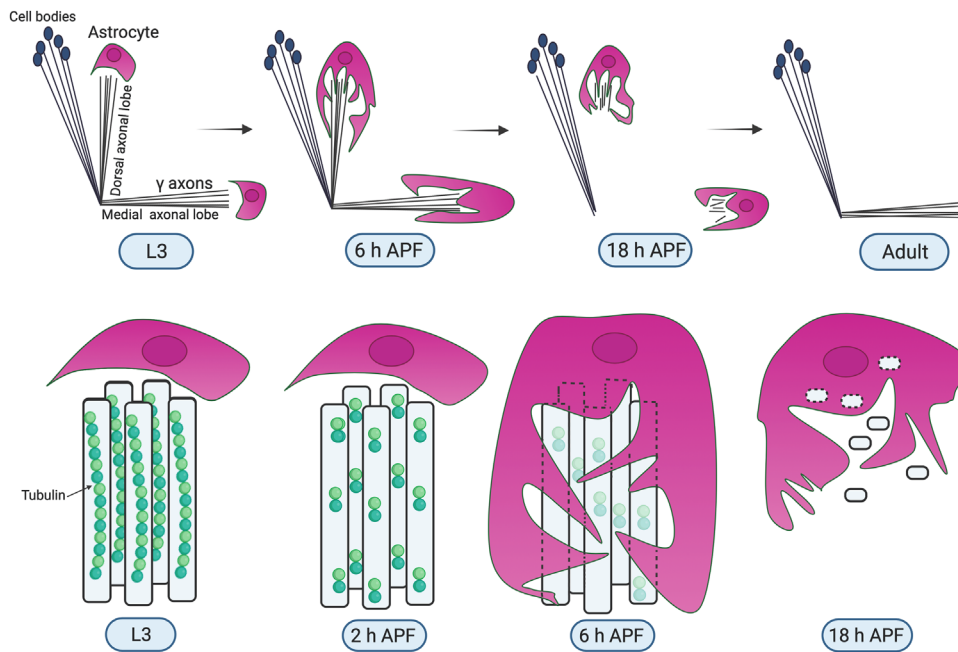
The central brain circuit *Pigment-dispersing factor* tritocerebral (Pdf-Tri) neurons are developmentally transient and are normally eliminated following hatching of the adult. It was suggested that these neurons have a hatching-related role within the clock circuit.<sup>[52]</sup> The Fragile X syndrome (FXS) is a leading cause of intellectual disability and autism spectrum disorder; the *Drosophila Fmr1* gene is an RNA binding protein whose loss causes FXS-related phenotypes. Fmr1 is required for the pruning of the Pdf-Tri neurons as evidenced by their inappropriate persistence in 5-day posteclosion mutant adults. It was recently shown that Fmr1 is expressed and required in Pdf-Tri neurons for normal pruning and that ensheathing and cortex glia are the major phagocytes involved in this process. Neuronal Fmr1 acts via the glial InR likely to promote the *drpr* transcription. The authors suggest that Fmr1 may regulate Ilps release via dense core vesicle exocytosis although the direct role of the Ilps as well as the link between Fmr1 and Ilps, in this process, were not tested.<sup>[53]</sup>

## Adult brain neurons signal glia via sApp1

In mammals, sAPP signals through an autocrine pathway to the neuronal death receptor 6 (DR6) and is involved in naturally occurring developmental neuronal pruning and neuronal cell death.<sup>[54]</sup> Interestingly, a recent study has shown that *Drosophila Appl* loss-of-function results in accumulation of dead neurons in the brain during a critical period of young adulthood. In addition, the extracellular domain of Appl is also secreted by neurons and taken up by glia through an unknown glial receptor. Therefore, sApp1 is proposed as a signal for neuron-glia communication and glial clearance of dying neurons in adult flies.<sup>[39]</sup> The role of *Drosophila Appl* in developmental neuronal pruning remains to be evaluated.

## ROLE OF THE CHEMOKINE-LIKE ORION ON MUSHROOM BODY REMODELING DURING METAMORPHOSIS

Mushroom bodies are prominent structures in the *Drosophila* brain involved in olfactory learning and memory.<sup>[55,56]</sup> Probably, the best documented case of neuronal remodeling is the developmental axon pruning of mushroom body  $\gamma$  neuron axons (MB  $\gamma$  axons) that occurs during metamorphosis.<sup>[2,6,57,58]</sup> Below we summarize the key different molecular and cellular events that occur during this complex process before describing the role of the recently identified chemokine-like Orion in the MB  $\gamma$  axons pruning. The  $\gamma$  neurons are the first to arise in the late embryo/newly hatched larvae and cease to be produced at the end of the L3 larval stage, in the developing MB. Larval neurons have branched axons projecting into both dorsal and medial lobes. At the beginning of metamorphosis,  $\gamma$  neurons undergo pruning of larval-specific dendrites and axons, which is followed by regrowth of adult-specific neurites. Notably, the adult MB  $\gamma$  axons do not bifurcate and therefore only project into the single medial lobe (Figure 3).<sup>[59]</sup> Most effects of the steroid 20-hydroxyecdysone (ecdysone), the hormone of metamorphosis, are mediated through the ecdysone receptor *EcR-B1* which is compulsory for  $\gamma$  axon pruning.<sup>[60]</sup> Precisely orchestrated neuron-glia crosstalk ensures the efficacy of  $\gamma$  neuron remodeling. First, the TGF- $\beta$  ligand Myoglianin (Myo), secreted predominantly from the cortex glia, activates the TGF- $\beta$  pathway through its receptors.<sup>[61]</sup> The TGF- $\beta$  receptor complex includes the type I receptor Babo and either one of the type II receptors Put or Wit. A novel immunoglobulin superfamily protein named Plum facilitates the signaling of the TGF- $\beta$  receptor complex likely by regulating the availability of the Myo ligand to the canonical receptor.<sup>[6]</sup> Then, the TGF- $\beta$  pathway together with the cohesin complex and the nuclear receptor pathway upregulate *EcR-B1* transcription within the larval  $\gamma$  neurons in a cell-autonomous fashion.<sup>[61-64]</sup> Potential candidate direct or indirect targets of *EcR-B1* within the  $\gamma$  neuron are the ubiquitin proteasome system (UPS) and the  $\alpha$ Tub ( $\alpha$ -Tubulin) gene.<sup>[65-67]</sup> UPS proteins are required for  $\gamma$  axon pruning and expression of an  $\alpha$ Tub-*myc* construct demonstrated that the microtubule cytoskeleton disappears by 8 h APF before the first signs of degeneration are observed. In brief, a cell-autonomous cascade within the  $\gamma$  neuron ensures axon fragmentation. Seminal studies have shown that MB surrounding glial extensions approach and penetrate to both  $\gamma$  lobes in the early pupa and engulf the axonal debris. Importantly, perturbing glial phagocytic functions delay the debris clearance. Moreover, it was clearly shown that altering ecdysone signaling, with *EcR-DN*, specifically in these glial cells results in a partial axon pruning defect in addition to a strong defect in debris clearance.<sup>[68]</sup> This was the first clear indication that MB surrounding glial cells may have a direct role, in some MB  $\gamma$  axons, in the process of axon pruning itself. Nevertheless, it was already proposed that glia have likely an active role in the remodeling process rather than simply just scavenging already degraded debris.<sup>[69,70]</sup> Importantly, the engulfment and phagocytosis by glial cells of the MB  $\gamma$  axons depends on *EcR-B1* function in the  $\gamma$  neurons. Although glia could just be creeping up the debris trail,



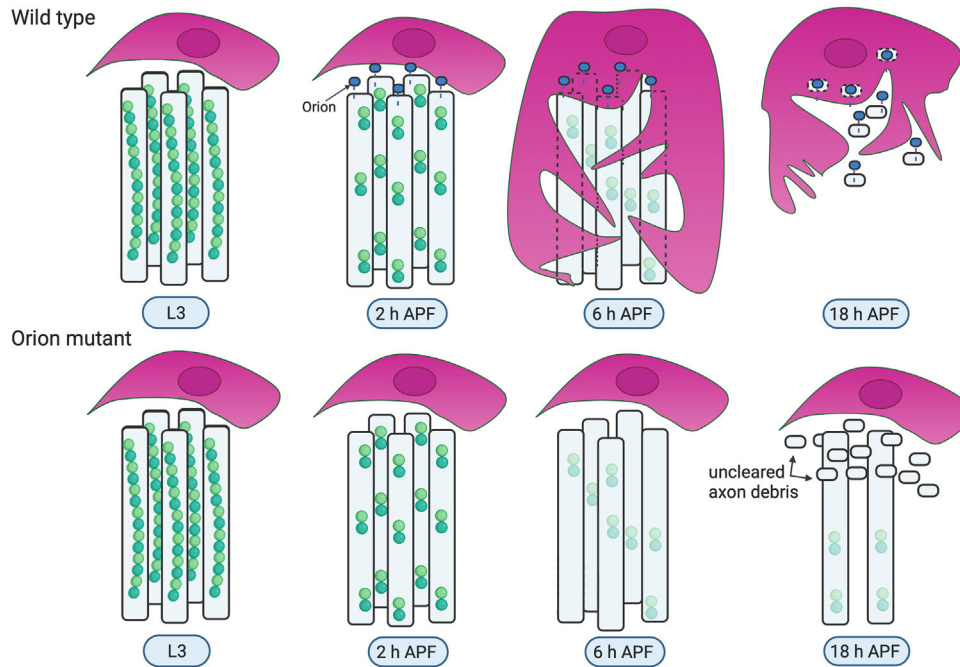
**FIGURE 3** Mushroom body  $\gamma$  neuron axon pruning in *Drosophila*. (Upper panel) During L3,  $\gamma$  axons are surrounded by astrocyte glia. At 6 h after puparium formation (APF) astrocytes infiltrate into both dorsal and medial  $\gamma$  bundles and phagocytose degenerating axons. The phagocytic process is mainly finished by 18 h APF and is followed by a final regrowth of axons into the unique bundle observed in adult flies. (Lower panel) Tubulin (green beads) disorganization is an early step of axon degeneration and starts at 2 h APF in an enlargement of the dorsal bundle. Progression of phagocytic steps occur in synchrony with the microtubule cytoskeleton disassembly

this could suggest that a neuronal signal might be produced by the MB  $\gamma$  axons in order to recruit glia around the  $\gamma$  lobes.<sup>[67,69]</sup> Finally, it was also shown that the major glial subtype responsible for clearance of MB debris are astrocytes.<sup>[22,68]</sup>

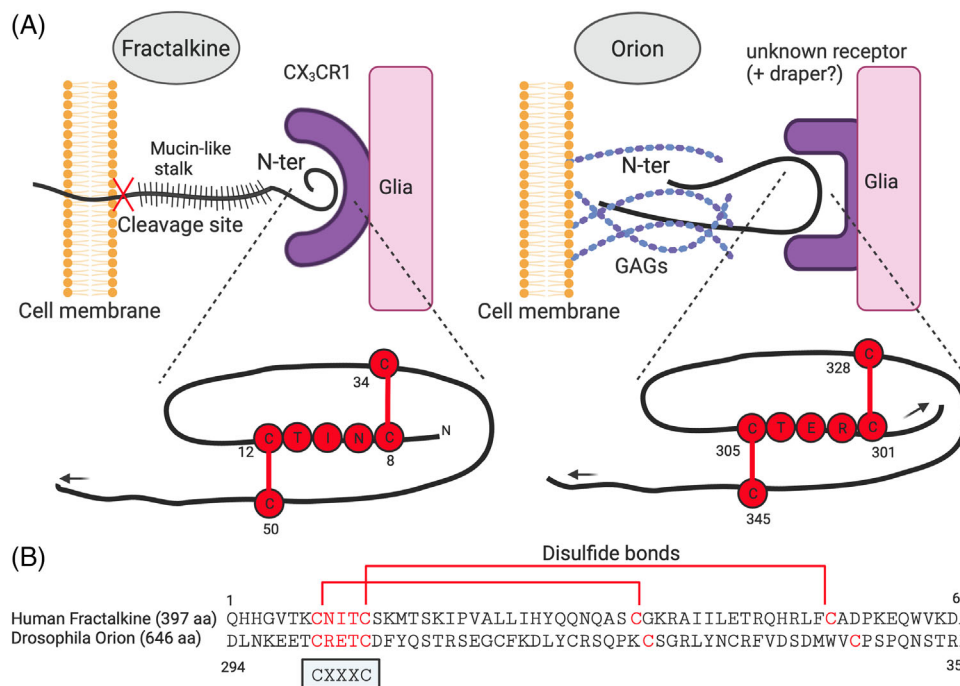
This neuronal signal is likely encoded by the newly identified gene *orion*.<sup>[71]</sup> A viable null mutation in the *orion* gene was found in a screen for defects in the adult MB axon pruning. Orion is secreted from the MB  $\gamma$  neurons, remains near the axon membrane, where it associates with infiltrating astrocytes, and is necessary for astrocyte infiltration into the  $\gamma$  bundles. In 6 h APF individuals bearing null alleles of *orion*, unlike in the wild-type control, there is essentially no glial cell invasion in the  $\gamma$  bundle. It appears that glial cells never infiltrate MBs in mutant individuals because glial infiltration as well as engulfment of the degenerated axons was not observed even at 24 h APF (Figure 4). The axon fragmentation is under the control of the MB intrinsic  $\gamma$  neuron program which is unaffected in *orion* mutant. The significant amount of axonal debris seen in adult *orion*<sup>null</sup> individuals is due to the failure of the astrocytes to clear the debris left from axon fragmentation. Nevertheless, in those individuals, some unpruned vertical MB  $\gamma$  axons persist, although the majority of axons degenerate, indicating that astrocytes have some function in axon severing. This is in accordance with the previous decoupling of two astrocytic processes: axon fragmentation and the subsequent clearance of axonal debris.<sup>[68]</sup>

Orion is expressed in  $\gamma$  neurons and is likely a transcriptional target of *EcR-B1*<sup>[25,72]</sup> in accordance with the previous hypothesis that a diffusible factor is produced by the MB  $\gamma$  axons in order to recruit glia around the  $\gamma$  lobes.<sup>[67,69]</sup> Orion is required in  $\gamma$  neurons but not in glial

cells for MB neuronal remodeling.<sup>[71]</sup> Orion is a secreted protein and bears some chemokine features such as a CX<sub>3</sub>C motif and three glycosaminoglycan (GAG) binding consensus sequences that are required for its function. Chemokines are a family of chemoattractant cytokines characterized by a CC, CXC, or CX<sub>3</sub>C motif promoting the directional migration of cells. Mammalian CX<sub>3</sub>CL1 (also known as fractalkine) is involved in neuron-glia communication.<sup>[8,73–76]</sup> Fractalkine and its receptor, CX<sub>3</sub>CR1, have been recently shown to be required for post-trauma cortical brain neuron microglia-mediated remodeling in a whisker lesioning paradigm.<sup>[24]</sup> Human fractalkine displays intramolecular disulfide bonds that appear to be conserved with respect to their distance from the Orion CX<sub>3</sub>C motif indicating the possibility of the conservation of a higher order CX<sub>3</sub>C domain structure (Figure 5). The CX<sub>3</sub>C domain is likely relevant for the binding of the fractalkine to its receptor.<sup>[77–79]</sup> Although bearing a CX<sub>3</sub>C motif that is essential for its function, Orion is much larger than typical vertebrate chemokines and is therefore unlikely to be a true fractalkine ortholog. Nevertheless, we propose that the binding of Orion to its unknown glial receptor could be, as in fractalkine, via its CX<sub>3</sub>C motif (Figure 5). The glial receptor *drpr* seemed also to be an obvious candidate based on the similarities between *orion* and *drpr* MB mutant phenotypes.<sup>[22,67,68,71]</sup> Nevertheless, the mutant phenotypes in *orion*<sup>1</sup> and *drpr* <sup>$\Delta$ 5</sup> are different with the *orion* mutant phenotype being more robust and essentially completely penetrant while there are only few unpruned axons in *drpr* mutant adults with a low penetrance.<sup>[68,71]</sup> This suggests that Drpr is not an, or at least not the sole, Orion receptor. Thus, Orion and its astrocyte receptor might be involved in both axon fragmentation and



**FIGURE 4** Orion secretion allows astrocytic gliosis and engulfment. In wild-type flies, Orion secretion occurs at roughly the same time that the disassembly of the microtubule cytoskeleton (Tubulin as green beads). Orion (blue dots) is present extracellularly at 2 h after puparium formation (APF) allowing astrocytes to infiltrate and engulf degenerating  $\gamma$  axons at 6 h APF. Only some mushroom body  $\gamma$  neuron axon debris containing Orion bound to membranes are still present at 18 h APF. In *orion* mutant flies, the  $\gamma$  axon intrinsic degeneration process continues. However, astrocytes are unable to infiltrate and phagocytose the  $\gamma$  axon bundle and therefore, large amounts of axonal debris as well as unpruned axons are observed at 18 h APF



**FIGURE 5** Comparison of the protein structure, amino-acid sequence, cellular location, and receptor binding of the mammalian fractalkine and Orion. (A) Fractalkine (or CX<sub>3</sub>CL1) is a transmembrane protein containing a mucin-like stalk at the extracellular domain that can be cleaved. Fractalkine binds to a microglial CX<sub>3</sub>CR1 receptor. Orion is secreted and binds to extracellular matrix glycosaminoglycans (GAGs) and an unknown astrocyte receptor. Both proteins contain a CX<sub>3</sub>C motif and potential disulfide bonds between cysteines (red color) represented in the enlarged image. This motif is essential for receptor binding for the fractalkine and is essential for the function of Orion. (B) The CX<sub>3</sub>C motif (in red) and putative Orion disulfide bonds (brackets) are likely conserved between human fractalkine and *Drosophila* Orion

debris clearance. This dual phenotype is similar to the EcR-DN induced phenotype in astrocytes.<sup>[68]</sup> Drpr, in the other hand, might be involved mainly in debris clearance with only a minor role in axon fragmentation. Finally, Orion does not seem to induce the Drpr signaling pathway in astrocytes.<sup>[71]</sup>

When it comes to cell–cell interaction, MB remodeling can be summarized as two steps of neuron–glia crosstalk and a  $\gamma$  axon-intrinsic fragmentation program; in brief, glia1  $\rightarrow$  neurons  $\rightarrow$  glia2. The first step involves cortex glial cell signaling by secretion of myoglianin, which is received by  $\gamma$  neurons to initiate a cell-autonomous cascade leading to  $\gamma$  axon fragmentation. The second step is the secretion of Orion by MB  $\gamma$  axons which is received by astrocytic glia to initiate their infiltration and engulfment of the  $\gamma$  axon bundle. Thus, the glia orchestrates developmental neural remodeling not only by engulfment and phagocytosis of unwanted neuronal debris but also by enabling developmentally specified neuronal fragmentation.

## CONCLUSIONS AND OUTLOOK

Signals sent from dying neurons or neurites to be pruned during developmental processes or to be removed after trauma are received by appropriate glial or epidermal cells. After receiving these signals, glial or epidermal cells are transformed into phagocytes and infiltrate degenerating sites and engulf and clear neuronal debris through phagocytic mechanisms. Our current knowledge indicates that specific signaling pathways may be used by the different types of glia, for example: Orion/unknown receptor in astrocytes; Spz5/Toll-6 in the cortex glia; and Ilps/InR in the ensheathing and cortex glia. Whether the apparent specificity of glial cell/signaling pathways can be related to differing molecular mechanisms of the neurite pruning in healthy neurons versus in injury-induced degeneration is not currently known. Neuronal signals, receptors and pathways involved in activation of phagocytic cells in *Drosophila* are mostly conserved in mammals with the central role of the *drpr* receptor, homologous to the human *MEGF10* gene, being involved in all the neuronal clearance described so far. While its primary amino acid sequence indicates that the CX<sub>3</sub>C chemokine-like Orion is likely not a structural ortholog of the human fractalkine it seems probable that there is functional conservation of the signaling mechanism employed by fractalkine and Orion. This may indicate that chemokine-like involvement in neuron/glial cell interaction is an evolutionarily ancient mechanism. Finally, the links between the several glial signaling pathways involved in the clearance of neuronal debris and the phagocytic mechanisms, such as phagosome formation and maturation, remain largely unknown and need be further investigated.

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## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Data sharing not applicable – no new data generated.

## ORCID

Jean-Maurice Dura  <https://orcid.org/0000-0001-8296-9023>

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