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Programmed cell death acts at different stages of *Drosophila* neurodevelopment to shape the central nervous system

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Nervous system development is a process that integrates cell proliferation, differentiation, and programmed cell death (PCD). PCD is an evolutionary conserved mechanism and a fundamental developmental process by which the final cell number in a nervous system is established. In vertebrates and invertebrates, PCD can be determined intrinsically by cell lineage and age, as well as extrinsically by nutritional, metabolic, and hormonal states. *Drosophila* has been an instrumental model for understanding how this mechanism is regulated. We review the role of PCD in *Drosophila* central nervous system development from neural progenitors to neurons, its molecular mechanism and function, how it is regulated and implemented, and how it ultimately shapes the fly central nervous system from the embryo to the adult. Finally, we discuss ideas that emerged while integrating this information.

Keywords: apoptosis; *Drosophila*; neurodevelopment

Apoptosis shapes *Drosophila* neural development from the emergence of neuroectoderm in the embryo to the eclosing adult. The earliest indication of programmed cell death (PCD) during neurodevelopment is at embryonic stages 11–12 [1–3], when the first neurons and epidermal cells die [4,5]. Neuronal apoptosis then increases dramatically peaking at embryonic stages 16–17, when the ventral nerve cord (VNC) condenses [3] and the embryo produces its first twitching movements [1]. Although the observed amount of neuronal death differs from embryo to embryo [5], symmetry in neuronal PCD is often observed between the right and left sides of a single embryo [1], indicating that both deterministic and 'random' processes participate in the selection of surviving neurons.

Programmed cell death in *Drosophila* neural development comes in different flavors. It can be triggered by spatial, temporal, nutritional, hormonal, and metabolic signals; it can be regulated by Hox genes, Notch, and other signaling pathways; it can be mediated by one or more proapoptotic genes; and it can act at the stage of neural precursors, of newly born or of mature neurons/glia. What is clear is that dying is not trivial for a cell. Many layers of regulation exist that ensure

Abbreviations
AbdA, abdominal-A; AbdB, abdominal-B; ANT-C, Antennapedia complex; APF; after puparium formation; BX-C, Bithorax complex; CB, central brain; GMC, ganglion mother cell; IAPs, Inhibitor of Apoptosis Proteins; INP, intermediate neural progenitor; IPC, inner proliferation center; MB, mushroom body; NBRR, neuroblast regulatory region; $Notch^{ICD}$, Notch intracellular domain; OL, optic lobes; OPC, outer proliferation center; PCD, programmed cell death; pNR, procephalic neurogenic region; RHG, Reaper, Hid, Grim; Scr, sex combs reduced; tTFs, temporal transcription factors; VNC, ventral nerve cord.

both proper timing of death and effective killing without sacrificing specificity for the cells that should (or should not) die. Here, we review in detail these different levels of regulation during *Drosophila* neurodevelopment. After introducing the mechanisms of apoptosis (Section 'Mechanisms of apoptosis') and how the *Drosophila* central nervous system develops (Section '*Drosophila* neurodevelopment: from the embryo to the adult'), we describe in detail the occurrence and regulation of apoptosis in progenitors (Section 'Apoptosis in neuroblasts') and neurons (Section 'Apoptosis in neurons') throughout the different stages of *Drosophila* neurodevelopment. Finally (Section 'Emerging ideas'), we discuss emerging ideas and perspectives in the field.

Mechanisms of apoptosis

The effectors of the apoptotic process are the caspases, which are cysteine proteases that, at the onset of apoptosis, undergo a cascade of catalytic activation reactions. Seven caspases have been identified in *Drosophila*: the initiator regulatory caspases Dronc, Dredd, and Strica, and the effector caspases Drice, Dcp-1, Decay, and Damm [6–13]. Once activated, effector caspases cleave their cellular targets, including structural proteins and enzymes, disrupting DNA replication and cellular metabolism.

Apoptosis in *Drosophila* is initiated by the proapoptotic genes *reaper* (*rpr*), *head involution defective* (*hid*), *grim,* and *sickle* (*skl*), collectively known as RHG [14] (Fig. 1A). Although the four genes are clustered in a small genomic region on chromosome III (Fig. 1B), their protein sequences are unrelated, only sharing weak homology in the first 15 amino acids [15–17]. Deletion of *reaper*, *grim,* and *hid* (but not *sickle*) in the *def(3)H99* deletion (Fig. 1B) blocks developmental apoptosis, while overexpression of each gene is often sufficient to induce apoptosis in a caspase-dependent manner in both insects and mammals [18–26]. In the fly, three Inhibitor of Apoptosis Proteins (IAPs) have been identified—DIAP1, DIAP2, and Deterin [23,27]. IAPs bind to caspases and inhibit their activity [28,29]. Part of the RHG proteins' proaptotic activity is due to their ability to bind and inactivate IAPs and in this way regulate caspase activity. In addition, Rpr and Grim have been shown to suppress DIAP1 translation [30,31] (Fig. 1A).

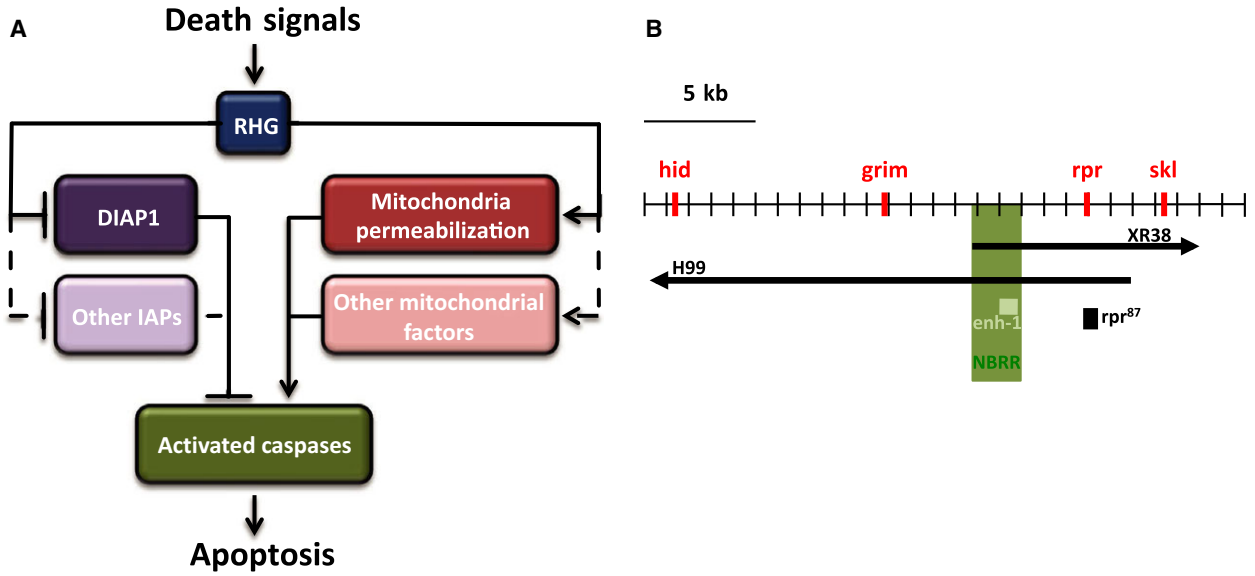

Fig. 1. Reaper, Hid, Grim, and Sickle orchestrate apoptosis in *Drosophila*. (A) Reaper, Hid, Grim, and Sickle (RHG) integrate signals from different sources, and trigger apoptosis. RHG act through two independent pathways. They either inhibit DIAP1, and/or other inhibitors of apoptosis, or they act by promoting mitochondria permeabilization. Both pathways ultimately lead to the activation of caspases, cysteine proteases that implement the apoptotic program. Solid arrows represent proven interactions and dashed lines represent unproven interactions. (B) Schematic representation of the genomic locus of the third chromosome where RHG are located. The neuroblast regulatory region (NBRR) is situated between *rpr* and *grim* and is responsible for the integration of developmental signals that control *reaper, grim,* and *sickle* expression, and, hence, apoptosis. A cis-regulatory element (enh-1) is responsible for the restricted expression of the proapoptotic genes in the abdominal neuroblasts. Three main genomic deletions that have been used to study apoptosis in flies. In deficiency *Df(3l)H99, rpr*, *hid,* and *grim* are removed, while in *Df(3l)XR38, rpr* and *skl* are not present. Finally, rpr^{87} only removes *rpr*. Black arrows represent the deleted regions, and red bars the proapoptotic genes. The NBRR is represented in green and, within it, the enh-1 enhancer in light green.

The RHG activity is combinatorial and synergistic [17,32,33]. For instance, the expression of *hid* or *rpr* alone in the CNS midline glia is insufficient to induce apoptosis; however, when both genes are expressed, they trigger extensive glial apoptosis. The expression of *grim* alone is sufficient to induce midline glial apoptosis, but its activity is also synergistic with that of *rpr* and hid [34].

While RHG proteins share similar downstream mechanisms, they are not functionally equivalent to each other and they do not share the same activation pathways; *hid* expression and activity are negatively regulated both transcriptionally and post-translationally by the Ras/MAPK pathway, while the p53/DNA damage pathway and ecdysone receptor-mediated signaling directly regulate *rpr* expression [35,36]. As a consequence, RHG are differentially expressed in dying cells in response to different signals they are competent to receive. In both the embryo and adult CNS, *rpr* and *grim* are broadly expressed in dying cells while *hid* is limited to the dying midline glia and is also expressed in some cells that do not undergo PCD [21,37,38]. *sickle* was identified as a damage-responsive gene. Like the other RHG proteins, Sickle binds DIAP1 but neither its overexpression induces apoptosis nor does its removal prevent it [16,17,32,33]. Instead, *sickle* activity appears to potentiate the activity of the other RHG genes [17,32]. The coordinated expression of RHG genes is thus crucial in regulating PCD, although it is still unclear how distinct combinations of RHG proteins impact IAP function in the fly. RHG transcription in neuroblasts is in part achieved by a NeuroBlast Regulatory Region (NBRR) that lies between the *rpr* and *grim* loci. This genomic region integrates multiple developmental signals to control the spatio-temporal pattern of apoptosis in neuroblasts through the expression of *grim*, *reaper,* and *sickle* [33] (Fig. 1B).

MicroRNA are also involved in apoptotic death regulation [39]. Different microRNA have been shown to act at different levels of the apoptotic cascade. Members of the evolutionarily conserved miR-2 family (miR-2/13, miR-6, miR-11, and miR-308) exert their antiapoptotic activity by downregulating *reaper, hid,* and *grim* [40]. Moreover, bantam, which encodes a microRNA with multiple roles during development, is able to regulate cell number by increasing cell proliferation and decreasing cell death by blocking *hid*-induced apoptosis during development [41,42]. The role of microRNA in regulating cell death during *Drosophila* neurodevelopment is yet to be shown.

During PCD, Rpr, Hid, and Grim localize to mitochondria [22,43,44]. Inhibition of Rpr or Hid mitochondrial localization prevents full caspase activation [44–46] suggesting a role for this organelle in the apoptotic process. In agreement with this, mutants for the gene encoding the dynamin-related protein Drp1, which regulates the rate of mitochondrial fission and fusion [47–49], show reduced mitochondrial fragmentation and decreased caspase activation [47]. Mitochondria are permeabilized in response to *reaper* and *hid* expression [45]. Interestingly, mitochondrial membrane permeabilization is independent of DIAP1 activity [45]. Hence, RHG may act in two independent pathways: (a) binding DIAP1 and relieving caspase inhibition and (b) permeabilizing mitochondria and increasing caspase activation (Fig. 1A). Altogether these observations show that that the survival of a cell is regulated by the interplay between RHG proapoptotic genes and the mitochondrial fusion machinery. Why mitochondrial localization of RHG proapoptotic proteins is significant for caspase activation, and what is the role of mitochondrial fragmentation and permeabilization remains unknown [50,51].

Drosophila neurodevelopment: from the embryo to the adult

The *Drosophila* central nervous system can be divided into optic lobes (OL), central brain (CB), and ventral nerve cord (VNC). In the embryo, the head neuroectoderm generates the brain structures while the ventral neuroectoderm gives rise to the VNC, composed of segmental units called neuromeres (three gnathal, three thoracic, and seven abdominal) [52–54] (Fig. 2A). Four additional segments and a nonsegmented telson constitute the most posterior part of the VNC ('tail region') [55]. The optic lobes develop later, during larval stages, from neuroepithelial placodes [53,56,57].

Neuroblast generation and proliferation

Drosophila neurogenesis starts at early stages of embryogenesis, with the specification of neurogenic versus non-neurogenic regions, determined by the early activity of the proneural genes and by Notch signaling [58–60]. In each equivalent group of neurogenic cells, one cell acquires a neuronal progenitor fate known as neuroblast and inhibits its neighbors that remain epidermal cells. Neuroblasts delaminate to the interior of the embryo [61,62]. Each neuroblast typically divides asymmetrically to self-renew and to produce a ganglion mother cell (GMC), which divides once, asymmetrically, to produce two postmitotic neurons or glia (neuroblast type I) [61]. During germ band elongation

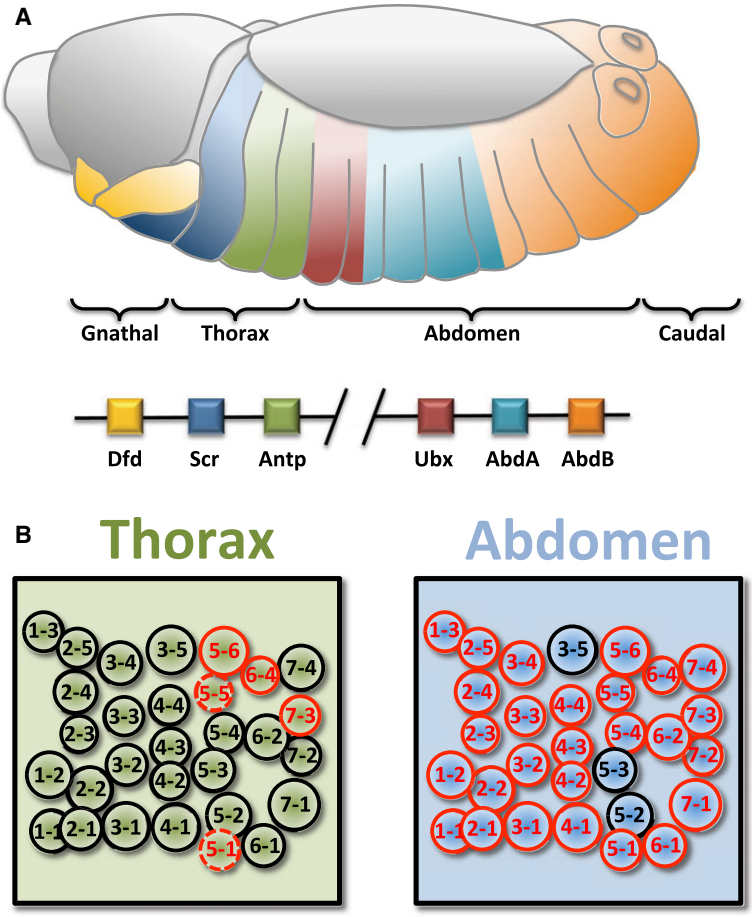

Fig. 2. Neuroblast fate is regulated by Hox gene expression and differs between abdominal and thoracic segments. (A) In the embryo, the identity of each segment depends on Hox gene activity. Sex combs reduced (Scr) and Antennapedia are active in the thoracic segments, while Ubx, Abdominal-A, and Abdominal-B are active in the abdominal and caudal segments, in a colinear manner with respect to their positions in the Hox cluster. The ventral nerve cord (VNC) is similarly subdivided into 17 neuromeres, 3 gnathal, 3 thoracic, 7 abdominal, and 4 caudal. (B) Upon delamination, 30 neuroblasts are observed in each thoracic and abdominal hemisegment. At the end of embryogenesis some thoracic and most of the abdominal neuroblasts are eliminated by PCD. When the larva hatches, 23 postembryonic neuroblasts per hemisegment are found in the thorax, 12 in A1, 4 in A2, and 3 in each of the A3–A8 abdominal hemisegments. Neuroblasts that are maintained to the larvae are labeled in black, apoptotic neuroblasts are labeled in red, and potential thoracic apoptotic neuroblasts are labeled in dashed red.

(stages 8–11), five sequential waves of neuroblast delamination in the embryonic VNC produce an invariant pattern of 30 neuroblasts per hemisegment [63–66] (Fig. 2B). Elegant heterotopic transplantation experiments between thoracic and abdominal sites of early gastrula neuroepithelium have shown that neuroblasts proliferate according to their domain of origin in a cell autonomous manner, demonstrating that segment-specific identity is programmed early during development at the level of the neuroepithelium [67,68]. Such a specification is established by the early expression of spatial patterning genes including the segment polarity genes [e.g., *runt*, *wingless* (*wg*), *gooseberry* (*gsb*)], which are stereotypically expressed in segmental stripes that subdivide each neuromere along the antero-posterior axis, and columnar patterning genes [e.g., *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*), *muscle specific homeobox* (*msh*)] that act along the dorso-ventral axis. The superimposition of these expression patterns establishes an almost invariant cartesian grid of positional information [69–72].

In each hemisegment, homologous neuroblasts under the same positional cues produce similar embryonic lineages [73–75]. However, homologous lineages do show some variations, in particular between thoracic and abdominal segments, reflecting the different requirements of each segment [73,75]. Segmental specificity is defined by the expression of Hox genes that act in the neuroepithelium, neuroblasts, neurons, and glia to control cell specification and proliferation/apoptosis in a segment-specific manner [71,76–79] (Fig. 2A). Hox genes from the Antennapedia complex (ANT-C) control the differentiation of the head, gnathal, and anterior thorax [80], while those from the Bithorax complex (BX-C) define the identity of the posterior thorax, abdomen, and caudal segments [81,82] (Fig. 2A).

As VNC neuroblasts age, they progress through a well defined 'series' of temporal transcription factors (tTFs) that dictate the identity of the neurons/glia produced by the neuroblasts based on their time of birth [83–88]. In the VNC, embryonic neuroblasts progress through the tTF series Hunchback (Hb) → Krüppel

(Kr) → POU domain proteins 1/2 (Pdm) → Castor (Cas) [85,87–90]. The temporal series continues during postembryonic VNC development, with postembryonic neuroblasts expressing Castor → Seven-Up (Svp) [91–93]. Interestingly, distinct sequences of transcription factors are used in different contexts [87,92,94,95], likely reflecting a universal strategy in the establishment of neuronal diversity. Thus, spatio-temporal patterns of neuroblast delamination, together with a neuroblast intrinsic tTF series define their unique identities and the lineages they produce (neuronal/glial types and cell number) [63,73,75,96,97]. A more derived, yet similar, neuroblast distribution pattern is observed in the three gnathal neuromeres that lie anterior to the thorax (28 neuroblasts per labial hemisegment, 26 in maxillary, and 22 neuroblasts in mandibular hemisegments) [98] and in the most posterior caudal neuromeres (30 in A8, 29 neuroblasts in A9, 11 neuroblasts in A10, and no neuroblasts in A11) [81].

The pregnathal segments that form the embryonic head give rise to the brain, which is structurally more complex than the VNC. It arises from a bilaterally symmetric procephalic neurogenic region (pNR). While most of the brain structures are formed during larval stages, the adult brain bauplan is laid out early during embryogenesis. About 100 neuroblasts per hemisphere are generated, each with a unique molecular identity [77,99–103]. A close inspection of the combination of markers expressed in brain neuroblasts and their relative positions suggests that several of them might be homogolous to VNC neuroblasts [63,98,103].

Ending proliferation

During embryonic neurogenesis, the larval CNS and about 10% of the adult neurons are produced. Before larval hatching, neuroblasts stop proliferating by either entering a quiescent state or by committing PCD. The majority of brain neuroblasts stop dividing by embryonic stage 14, with only one lateral neuroblast and the four mushroom body neuroblasts per hemisphere escaping quiescence and dividing throughout development until late pupae [104] (Fig. 3).

Neuroblasts in the VNC also stop proliferating at the end of embryogenesis. While most of the thoracic neuroblasts enter quiescence, the majority of gnathal and abdominal neuroblasts are eliminated by PCD [33,66,105–107] (Fig. 3). Thus, when the larva hatches, a total of 19 postembryonic neuroblasts are found in the gnathal segments, 23 postembryonic neuroblasts per hemisegment are found in the thorax, 12 in A1, 4 in A2, and 3 in each of the A3–A7 abdominal hemisegments.

Central brain and VNC neuroblasts resume proliferation during larval stages, triggered by increased levels of circulating amino acids as the first instar larva starts feeding [108–110]. It is during the larval and pupal periods that 90% of adult neurons are produced [66,111,112]. At the same time, the differences between serially homologous lineages become even more pronounced, matching the transition from a crawling larva to an adult fly with much more complex behaviors and different requirements for the different adult segments. Neurogenesis progresses until the pupal

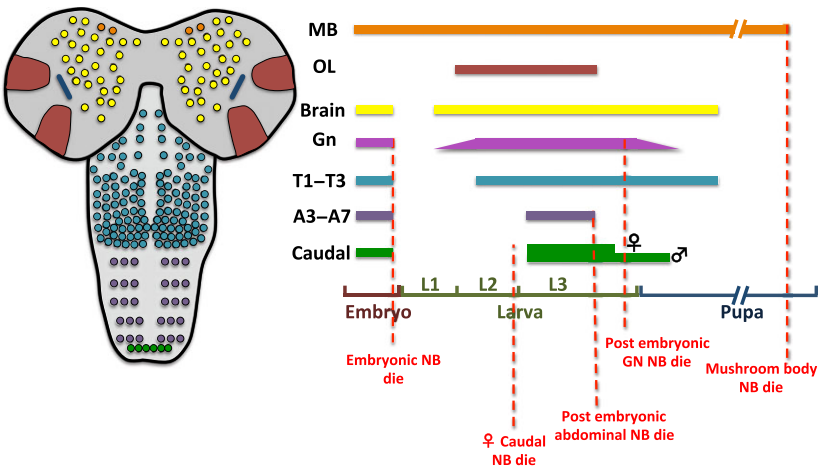

Fig. 3. Patterning of neuroblast activity and apoptosis during embryonic, larval and pupal development. Colored bars represent the mitotic activity of the neuroblasts depicted in the left cartoon (gnathal neuroblasts are not visible). Red dashed lines indicate the timing of neuroblast apoptosis. Note that gnathal neuroblasts division is approximate and deduced based on limited description in the literature (see main text). The timing of the female caudal neuroblast apoptosis and gnathal neuroblasts is approximate (see main text). Neuroblast number is representative and does not correspond to the real number. MB, mushroom body; OL, optic lobe; Gn, gnathal; T, thorax; A, abdominal.

stage, when postembryonic neuroblasts stop dividing either by undergoing a Prospero-dependent cell cycle exit (terminally differentiating into a GMC) or by PCD [93,106] (Fig. 3).

Apoptosis in neuroblasts

Embryonic neuroblast apoptosis

The mechanism by which most abdominal neuroblasts die at the end of embryogenesis was only recently elucidated. A cis-regulatory element (enh-1) was identified within the NBRR that restricts expression of RGH genes to abdominal neuroblasts [113] (Fig. 1B). This element is activated by a pulse of Abdominal-A (AbdA) at the end of embryogenesis. AbdA expression is the result of Notch being activated by the Delta ligand, which is expressed by the neuroblast's glial progeny. Therefore, it is the progeny of the neuroblast that induces its apoptosis, assuring its death only after it has produced the proper array of neurons and glia. Thus, Hox gene expression controls neuroblast apoptosis at two levels: early in embryogenesis by specifying the neuroblast's lineage, and at the end of embryogenesis by promoting its apoptosis. Upregulation of AbdA occurs upon ectopic overexpression of the Notch intracellular domain ($Notch^{ICD}$), but only in segments that expressed AbdA early in development. This suggests that early AbdA expression induces chromatin conformational changes that facilitate its later activation by Notch. Broad ectopic expression of AbdA only regulates RHG expression later in embryogenesis, and AbdA is also upregulated upon Notch ectopic expression only in late embryogenesis. This indicates that neuroblast competence to respond to both signals is established later during embryogenesis, preventing precocious neuroblast apoptosis.

Extensive neuroblast apoptosis is also observed at the end of embryogenesis in the gnathal segments (Fig. 3). While ~80 embryonic neuroblasts are observed at stage 12, only 24 are observed at stage 16 and ~19 in the first instar larva [105]. PCD also occurs at the level of the neuroectoderm. The Hox gene *deformed* regulates PCD in the neuroectodermal progenitors that constitute the neuroblast 6-4 proneural cluster of the gnathal maxillary and mandibular hemisegments, revealing yet another level where PCD acts to regulate cell number in the developing CNS [98].

Most of the head and thoracic neuroblasts exit the cell cycle and stop proliferating in a Prospero-dependent manner in the pupae. Exceptions are observed in the thorax; neuroblasts 5-6, 6-4, and 7-3 die at stage 16 [114,115]. Also, the lineages of neuroblasts 5-1and 5-5 differ between wild-type and *H99* deficiency clones [5,115,116]. Such differences might arise due to neuroblast or neuronal apoptosis.

Postembryonic neuroblast apoptosis

The end of neuroblast proliferation in the VNC occurs at different times in different regions, being complete by the end of metamorphosis [93,104]. In the central brain and thorax, most postembryonic neuroblasts cease dividing around 20 h after pupal formation [66]. Removing RHG activity does not prevent or delay loss of postembryonic neuroblasts, suggesting that they end proliferation by terminal differentiation [93]. This is not the case for abdominal postembryonic neuroblasts, which stop proliferation by apoptosis in the late larvae [67,83] (Fig. 3).

Ventral nerve cord

In postembryonic neuroblasts of the ventral nerve cord, tTFs (Hb → Kr → Pdm → Castor → Svp) not only specify neuronal identity but they also schedule the end of postembryonic divisions via Prospero-dependent cell-cycle exit for thoracic neuroblasts and via apoptosis for abdominal neuroblasts [93] (Fig. 4A). Castor simultaneously promotes Grainyhead (Grh) expression and the downregulation of Dichaete (D). This bestows neuroblasts with the competence to respond to a burst of AbdA in the late larvae. This intercepts the default terminal differentiation program and promotes apoptosis (Fig. 4A). If Grh expression is repressed or D is not silenced, AbdA cannot trigger apoptosis. Removal of Svp or persistent Cas expression prevents abdominal neuroblast apoptosis, despite normal AbdA expression [93]. This suggests that postembryonic neuroblasts have to transit through the temporal series to establish the competence to die when they assume the D^- Grh^+ Cas^- $AbdA^+$ code. Therefore, expression of tTFs induces and maintains the activation or repression of downstream targets (such as Dichaete and Grainyhead), allowing aging postembryonic neuroblasts to acquire specific tTF codes that regulate the timely death of postembryonic neuroblasts [93]. The establishment of neuronal identity and neuronal number is thus tightly linked to the tTFs. The recurring implementation of tTFs in establishing neuronal identity in multiple contexts may also regulate the aging of neuroblasts and end of their divisions.

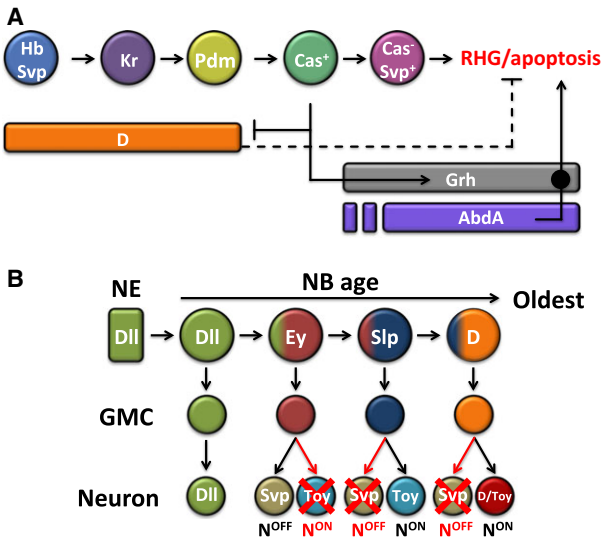

Fig. 4. Neuroblast tTFs control apoptosis in both neuroblasts and neurons. (A) Abdominal neuroblasts have to transit trough the tTFs to schedule apoptosis. The tTF Castor downregulates the expression of Dichaete that inhibits precocious RHG activation and upregulates Grh. Grh installs the competence to respond to a pulse of AbdA that triggers apoptosis. (B) Neuroblasts tTFs controls Notch-mediated neuronal apoptosis. Two pathways determine the binary life-or-death fate of neurons. tOPC neuroblasts tTFs control both the identity of the neurons produced at each time window and their survival by specifying the death of $Notch^{ON}$ neurons in a first phase and of $Notch^{OFF}$ neurons in a second phase. The red arrows indicate the dying neurons.

Polycomb group genes are required for postembryonic neuroblast survival and their removal promotes Hox gene expression and postembryonic neuroblast apoptosis in the central brain, as well as in the ventral nerve cord, thorax, and abdomen [117]. How are Polycomb genes regulated in postembryonic neuroblasts and what are their target genes? One possibility is that they act downstream of the tTF series, which could trigger the timely expression of AbdA. Another possibility is that, like in the embryo, AbdA expression is dependent on signaling from the postembryonic neuroblast progeny, which can act by regulating Polycomb gene expression. Supporting this hypothesis is the fact that, like in the embryo, the overexpression of Notch ^{ICD} in larval neuroblasts triggers AbdA expression and promotes apoptosis [113].

A conundrum arises however, since at the end of embryogenesis, most of the abdominal neuroblasts die after a pulse of AbdA (see previous section) although they have not transited through the full temporal series and established the D^- Grh^+ Cas^- $AbdA^+$ code. Interestingly, ectopic expression of either AbdA or the thoracic Hox genes Antp and Ubx proteins can induce apoptosis of both thoracic and abdominal postembryonic neuroblasts. This suggests that all Hox genes have the ability to induce postembryonic neuroblast apoptosis [118]. Hox-dependent neuroblast apoptosis is also observed in the gnathal segments in the embryo and early larvae [105]. From the initial 80 neuroblasts, 24 are observed in the stage 16 embryo, 19 in the first instar larvae and 14 in the third instar larvae. Unlike abdominal neuroblasts, thoracic embryonic and postembryonic neuroblasts do not express any Hox gene, and therefore do not undergo apoptosis. Could this expression be dependent on the amount of signaling given by postembryonic neuroblast progeny as is observed in embryonic abdominal neuroblasts? A closer inspection and comparison of the lineages of thoracic, apoptotic, and nonapoptotic abdominal and gnathal neuroblasts might uncover a relationship between the amount/type of progeny/signaling with neuroblast Hox gene expression and PCD activation.

The four posterior segments of the embryonic abdomen constitute the caudal region: a set of four postembryonic neuroblasts, two per side, exhibits sex-specific proliferation. In the female, these postembryonic neuroblasts stop proliferating in the midthird instar larvae, but the same neuroblasts continue dividing in the male during the larval and pupal stages producing a sex-specific lineage [66]. Such a specification is regulated by the activity of the gene *doublesex* (*dsx*), which encodes a transcription factor controlling male or female differentiation [119]. Interestingly, *dsx* plays a dual role in sex-specific neuroblast fate: the Dsx female isoform promotes neuroblast PCD while the male isoform is required for neuroblast survival [81] (Fig. 3). Neuroblast survival is also partially dependent on the activity of Abdominal-B (AbdB), but the mechanisms by which this gene and other factors regulate neuroblast sex-specific survival remain to be clarified.

Head

In the central brain, eight additional neuroblasts, known as type II neuroblasts, can be found. Type II neuroblasts divide asymmetrically, self-renewing and producing an intermediate neural progenitor (INP), which will divide three to five times to self-renew and produce a GMC [120]. Both type I and type II neuroblasts in the central brain stop dividing by a terminal symmetric division. The same is also true for INPs [93,121].

Optic lobe neuroblasts start to be produced during the larval stages and neurogenesis lasts until early pupa [53,122–126]. Neuroblasts originate from the inner (IPC) and outer proliferation center (OPC), two

neuroepithelial crescents that generate most of the neurons and glia populating the lamina, medulla, lobula, and lobula plate neuropiles [94,95,127,128]. OPC neuroblasts terminate proliferation by terminally differentiating [95] (Fig. 3), but no studies have yet addressed how IPC neuroblasts proliferation is stopped.

Mushroom body (MB) neuroblasts are the longest active neural stem cells in the fly; they start proliferating in the embryo and continue uninterrupted until midpupa, when they stop proliferating through apoptosis [129] (Fig. 3). Curiously, in transheterozygous flies for *Df(3l)H99* and *XR38* (a deletion that removes *rpr* and the NBRR), MB neuroblasts persist until later stages and keep proliferating, producing neurons that are incorporated into the adult mushroom body structure. Interestingly, however, even in the absence of this RHG activity, mushroom body neuroblasts are still later eliminated. This happens because prior to elimination, a reduction of insulin/PI3Kinase signaling triggers neuroblast autophagy. In this way a fail-safe mechanism to ensure MB-neuroblasts elimination by promoting neuroblast autophagy is installed. In the absence of FOXO, RHG-dependent apoptosis is delayed, suggesting that FOXO regulates both the timing of RHG-dependent apoptosis and the autophagy fail-safe mechanism. It further suggests that the timing of elimination of MB neuroblasts depends on the nutritional status of the animal (see also [130].

Apoptosis in neurons

Embryonic neuronal apoptosis

Many neurons are destined to die, unless they receive intrinsic or extrinsic survival signals. A characteristic example is that of pioneer neurons and the midline glia in stage 13 embryos. The segmentally repeated early differentiating neurons pCC, MP1, dMP2, and vMP2 extend their axons to delineate the major axonal tracts that span the embryo on either side of the midline [131–133]. These pioneer neurons express two EGF ligands, Vein, a homolog of the vertebrate Neuregulin, and Spitz, which promotes the survival of the neighboring midline glial cells that help fasciculate the axons and form the longitudinal bundles and the commissures that cross the midline in every segment [134,135]. Glial cells compete for EGF ligand, and those that fail to activate the EGFR pathway express the proapoptotic gene *hid*, triggering apoptosis [136,137]. In turn, midline glial cells regulate the survival of follower neurons that will constitute the longitudinal axon tracts, forming a feedback loop between neurons and glia [138].

After forming the longitudinal axonal pathways, some of the pioneer neurons die in a Hox-dependent manner. Posterior MP1 and dMP2 neurons survive to larval stages, due to the AbdB-mediated repression of *grim* and *reaper*. However, their anterior counterparts undergo PCD, which can be rescued by mis-expression of AbdB [139]. Interestingly, the surviving posterior dMP2 neurons assume a different role during larval stages, as they 'transdifferentiate' into ilp-7-expressing neuroendocrine neurons that innervate the hindgut [140]. Similarly, MP1 neurons become pdf-expressing peptidergic interneurons at larval stages [141]. The exact opposite apoptotic pattern can be observed in the MP3 grasshopper pioneer neurons. Thoracic MP3 neurons survive, while those in abdominal segments A3–A6 become obsolete after guiding the follower neurons, and undergo apoptosis [3]. The contribution of Hox genes has not been shown rigorously in this case.

Contrary to the antiapoptotic role that AbdB plays in the midline pioneer neurons, it is capable of exerting a proapoptotic role in other contexts during embryonic neuronal development. For example, the Capability neuropeptide-expressing Va neurons survive only in anterior abdominal segments A2–A4, while they undergo AbdB-driven apoptosis in the posterior segments [142].

Most of the neuronal cell death that is observed during *Drosophila* embryonic development occurs during stages 14–17, when the ventral nerve cord condenses by almost a quarter of its length [143]. In parallel, a massive apoptotic wave spanning stage 13 to stage 17 decreases the glial cells number by almost 75% [134,137,144], although the extent of glial death in the later stages has been challenged [5]. Initially, apoptotic cell death is observed uniformly in the ventral nerve cord, gradually being restricted to the anterior and posterior termini. By stage 17, more than half of neuronal apoptosis occurs in the posteriormost segments A6–A8 [143]. Forcing neurons, glia, or neuroblasts to survive during this extensive phase of apoptosis by expressing the baculovirus protein p35, which acts as a broad caspase inhibitor and interrupts programmed cell death, inhibits condensation of the VNC, but does not appear to have detrimental effects for the survival of the adult fly [143] (discussed further in Section 'Forcing dying cells to survive').

Aside from their role in neuronal survival, glial cells act as the macrophages of the CNS by removing neuronal debris. They are activated after neuronal PCD [145–147]. A number of phagocytic receptors, such as Draper and Six-microns-under (Simu), are expressed in

embryonic glia and participate in the phagocytosis of apoptotic neurons [148,149].

Apoptotic death occurs both in irregular and in segmentally repeated patterns. One lineage that has been studied extensively for its very stereotyped apoptosis is that of the ventral nerve cord neuroblast 7-3 [150–153]. In this lineage, the first GMC gives rise to the EW1 serotonergic interneuron and the GW motoneuron; the second GMC generates the EW2 serotonergic interneuron and a neuron that undergoes PCD; the third GMC generates the EW3 corazonin-positive (vCrz) interneuron and its apoptotic sibling. All these binary cell fate or survival decisions are mediated by the asymmetric inheritance of Numb in one of the neuronal progeny, which inhibits Notch signaling in this cell. If the sibling of EW3 that undergoes PCD is forced to survive, it also differentiates as a vCrz interneuron [150–152,154].

Neurons that do not undergo apoptosis following a Notch-mediated binary cell fate decision are often subject to Hox-mediated, segment-dependent apoptosis. In the case of the ventral nerve cord neuroblast NB7-3, the GW motorneuron that was generated by the first GMC, undergoes apoptosis in segments T3–A7. Ubx, which is expressed in these segments, is necessary and sufficient to induce *reaper* and kill the motorneurons [5,153].

Larval neuronal apoptosis

During larval stages, the extent of neuronal apoptosis is very restricted compared to the embryo. Most of the neurons that undergo PCD in the larva die immediately after the division of the GMC. Hemilineages in which half of the neurons die in a Notch-dependent manner are produced extensively by central brain neuroblasts. The 100 neuroblasts that populate each brain hemisphere are responsible for the production of about 100 000 neurons [155]. An example of such binary choices is observed in the four engrailed-expressing neuroblast lineages of the central brain, MC1, MC2, AC, and PC. GMCs produced by the MC1 neuroblasts give rise to two different neurons, while AC and PC neuroblasts generate only one neuronal type that survives ($Notch^{OFF}$) and one that dies via apoptotis ($Notch^{ON}$). Contrary, in the case of the MC2 neuroblasts, the $Notch^{ON}$ progeny survives while the $Notch^{OFF}$ undergoes apoptosis [156]. A similar mode of neurogenesis where one of the two progeny of the GMC dies in a Notch-dependent manner can be observed in more than 25% of the central brain lineages [157–159], in some lineages of the optic lobe [94], as well as the ventral nerve cord [160,161]. It is important to note the essential contribution of the tTFs in regulating neuronal death. In the tips of the OPC of the developing optic lobes, tTF expression in the neuroblasts modulate whether their $Notch^{ON}$ or $Notch^{OFF}$ neuronal progeny die in a specific temporal window. More specifically, they regulate the expression of proapoptotic genes to trigger the apoptosis of $Notch^{ON}$ (*reaper*) or $Notch^{OFF}$ (*hid*) neurons [94] (Fig. 4B). The same regulation might occur in hemilineages of the central brain and of the VNC.

Pupal neuronal apoptosis

Metamorphosis has given holometabolous insects the opportunity to inhabit different environments at different life stages. However, this comes at a cost, as these insects need to develop two different nervous systems to accommodate the different needs of the larval and adult life stages. During metamorphosis, larval neurons can be largely divided into two categories: those that die and those that prune and remodel their dendrites and axons to acquire a new function in the adult nervous system [e.g., mushroom body neurons [162]]. Neurons undergo apoptosis in two waves. Larval neurons that are not retained in the adult and are not needed for ecdysis, the molting of the fly's exoskeleton, undergo PCD a few hours after pupal formation. The neurons that participate in ecdysis die after metamorphosis within 24 h after eclosion [163,164].

20-hydroxyecdysone is a steroid hormone that regulates metamorphosis and ecdysis in arthropods and plays a central role in the control of apoptosis of larval neurons. Three ecdysone pulses regulate larval to pupal to adult transitions: a 'late larval pulse' at puparium formation, a small 'prepupal pulse' 10 h after puparium formation (APF), and a long 'pupal pulse' starting 24 h APF that decreases gradually until eclosion [165]. Ecdysone acts through its hormonal receptors, EcR-A, EcR-B1, and EcR-B2 to differentially regulate *grim* and *reaper* expression in different neurons and induce apoptosis [37,166].

Soon after the late larval pulse of ecdysone (6 h APF), Corazonin-expressing peptidergic interneurons (vCrz) undergo apoptosis [167]. PCD of vCrz neurons is triggered by ecdysone via its receptors, EcR-B1 and EcR-B2, and is mediated by *reaper* but surprisingly not DIAP1. After the small prepupal pulse, RP2 motorneurons in abdominal segments A2–A7 are eliminated; this process also requires the B isoforms of the ecdysone receptor and *reaper*, and is also independent of DIAP1 activity [168]. Interestingly, although RP2 motorneurons in A1 and aCC motorneurons in A2–A7 express EcR-B isoforms during the prepupal pulse,

they survive into the pupal stage [168]. These two observations suggest an unknown additional layer of PCD regulation besides ecdysone.

A number of optic lobe neurons also die in late larval and pupal stages in a complex spatiotemporal pattern [169]. Spatially, two distinct clusters of cells undergo apoptosis in the lamina, four clusters in the medulla and one cluster in the lobula plate and the region of T2/T3/C neurons between the lobula plate cortex and the medulla rim. Temporally, neuronal apoptosis spans the entire pupal stage in the optic lobe, with most of the affected optic lobe neurons dying soon after the prepupal pulse in an ecdysone/EcR-B1-dependent process [170].

While ecdysone triggers apoptosis in vCrz, RP2 neurons, and several optic lobe neurons, it is required for the survival of other neurons during the long pupal pulse. Approximately 300 ventral nerve cord neurons, termed type II neurons, express high levels of the ecdysone receptor EcR-A during metamorphosis. These neurons remain alive until eclosion and degenerate once ecdysone levels fall [37,171]. Their apoptosis can be reversed by the administration of ecdysone or by decapitation. Ecdysone treatment inhibits the expression of *reaper*, while decapitation leads to the accumulation of low levels of reaper that are not sufficient to trigger apoptosis [37,171]. Ecdysone acts on type II neurons by inhibiting the expression of *reaper* and *grim*, preventing neurons from undergoing PCD. At the same time, CCAP-expressing neurons die in an identical ecdysone decline-dependent manner [166,172].

Survival and apoptosis of different neurons during pupal stages can also be sex-specific. *fruitless* is alternatively spliced in males and females, and a specific male isoform acting in male neurons is necessary for the specification of sex-specific neuronal circuits and for male courtship behavior [173]. *fruitless* is involved in the selective apoptosis of the male-specific mAL neurons in the female, which is mainly driven by *reaper* [174].

Emerging ideas

Regulation of apoptosis

If there is one rule for PCD during neurodevelopment, it is that there is no rule. PCD emerges under different circumstances, and may be driven by different regulators. Apoptosis can be triggered by both intrinsic and extrinsic signals, and may utilize one or a combination of the aforementioned proapoptotic genes, *reaper*, *hid*, *grim*, and *sickle*.

The intrinsic regulators can be either temporal factors, which signal timely apoptosis of neuroblasts or neurons, or spatial signals that instruct cell fate based on their location. The most widely used temporal signals in the CNS operate in neuroblasts—as neuroblasts progress through the tTFs, competence to terminally differentiate or die is installed. However, not all neuroblasts have the same fate, which is to a large extent defined by their location and is under Hox regulation. The same is observed in neurons—the survival of the midline pioneer neurons, MP1 and dMP2, depends on the expression of AbdB [139]. An additional level of PCD regulation relies on a Notch-dependent binary cell fate decision in the hemilineages that are generated from GMCs. In many cases during larval neurogenesis of adult neurons, one of the two neuronal progeny of the GMC undergoes Notch-dependent apoptosis, which in some cases results in the $Notch^{ON}$ neuron or in other cases the $Notch^{OFF}$ neuron dying. Finally, sex-specific PCD regulates both neuroblast and neuronal fate.

To trigger apoptosis, temporal, spatial, and Notch-dependent signals have to activate the expression of one or more of the proapoptotic genes, *reaper, hid, grim*, or *sickle*. Their expression pattern differs widely during neural development, although three of them (*reaper, hid,* and *grim*) are able to trigger apoptosis when misexpressed. What is the individual role of these four genes? What is the selective advantage of having four different apoptotic regulators?

reaper and/or *grim* are required for almost all of the CNS apoptosis occurring in the embryonic stages, as well as for most of the postembryonic neuronal PCD, which is mostly mediated by ecdysone [18,38,139]. *hid*, on the other hand, which is widely used in non-neuronal PCD [175], is expressed more sparsely in embryonic neuronal tissues, and not exclusively in neurons that are destined to die. *hid* seems to be responsible for the death of midline glia, in collaboration with *reaper* [137,176]. *reaper* and *grim* are needed to trigger apoptosis of neuroblasts in the abdominal neuromeres [33]. *grim* appears to regulate apoptosis of neuropeptide-expressing neurons, such as the vCrz (in collaboration with *reaper*) [151,167], Capability-expressing Va neurons [142], and CCAP-expressing neurons [172]. It is also important to highlight that even if one of the RHG genes is not expressed in an apoptotic cell, it is often sufficient to trigger apoptosis if mis-expressed in this cell; for example, although *hid* and *reaper* are responsible for midline glia apoptosis, ectopic expression of *grim*, which is not expressed in these cells, can induce their apoptosis [177].

Another difference between the RHG genes is that their expression is regulated by different environmental conditions and by distinct signaling molecules. For example, the different ecdysone receptors have specific effects on the expression of *reaper*. Moreover, *reaper* expression is also regulated by the p53/DNA damage pathway [35,36,178], while *hid* expression is regulated by the EGFR and the Ras/MAPK pathway [179,180]. A plausible model that could explain the existence of four genes with similar downstream functions is that they are partially redundant and accumulation of proapoptotic activity is what irreversibly triggers apoptosis. By integrating information from different sources that differentially control each of them, the proapoptotic genes 'compute' whether a cell needs to undergo apoptosis. This secures cell death efficiency, timing, and specificity.

Forcing dying cells to survive

At different developmental stages, up to 30% of the cells of the CNS are dying. What happens when a cell that is supposed to die is forced to survive? This is a fascinating question, albeit slightly arbitrary in its interpretation, as 'living dead' cells are normally not encountered in nature. There are two ways to approach this question. One is to assess neuronal identity and connectivity in mutants that lack RHG genes. In this case, the neuronal apoptotic program cannot even begin. A second method is to overexpress an exogenous protein, the baculovirus p35 protein, to prevent death after the death cascade has started. It has been reported that p35-expressing rescued neuroblasts appear larger than normal, probably because the apoptotic cascade has already initiated [181]. Third, one can force neuroblasts to survive, and assess the identity of neurons that should have never been produced. Although the interpretation of cell identity might be confusing in this case, as the neuroblasts have escaped PCD and, potentially, cell cycle control, 'immortal' neuroblasts and their progeny represent a great model for studying neural tumors [182].

Embryos homozygous for the H99 deletion, where cell death is inhibited, display an enlarged VNC often merged with the epidermis [143], and die as embryos. However, inhibiting apoptosis in neurons is mostly tolerated in flies. Some innervation defects are observed [183], but, in general, neurons that survive manage to form connections and participate in a hyperplastic nervous system [5,38,137,166]. For example, if the death of the anterior pioneer neuron dMP2 is prevented by overexpression of p35, the 'undead' neuron seems to follow the fate of its posterior counterpart and differentiate into neuroendocrine neurons, although the presence of AbdB appears necessary for specific aspects of terminal differentiation [139]. Aside from neurons, supernumerary midline glia in H99 embryos also seem to incorporate normally in the midline [144].

In many hemilineages neurons are eliminated even before differentiating. The reason why this happens is not understood. Perhaps the generation of these neurons is part of a conserved neurogenic program that included the generation of sibling neurons that may no longer be needed but may have been necessary in ancestors that lived in different environments and under different selective pressures. Another possibility is that the generation of more neurons than needed favors fast adaptation in the face of different environmental pressures. PCD may be activated or repressed in different neurons of different lineages, facilitating a rapid remodeling of the neuronal circuitry.

Another question that arises is what the identity of 'living dead' neurons. In the case of the hemilineages, where GMCs give rise to one living and one dead neuron, what identity will the dead neuron acquire if it is forced to survive? Will it be the same as its sister cell? The answer to this question is case-dependent. For example, the second GMC from embryonic NB7-3 gives rise to neurons EW2 and EW2sib, which undergoes cell death. If EW2sib is forced to survive, it expresses similar markers to its sibling EW2 (i.e., Kr and Zfh2), although this does not necessarily indicate identical fates [150]. Similarly, for neuroblasts NB2-1, NB2-2a, NB2-4a, NB2-5, NB3-1a, NB3-2, NB3-5, NB4-4, NB5-1, NB5-4a, NB5-5, NB6-1, and NB7-1, the additional neurons observed in H99 embryos that should have died project in an identical manner to their sibling cells. In contrast, in neuroblasts lineages NB4-2, NB5-3, NB7-2, and NB7-4, H99 embryos show not only additional cells but also irregular axonal projections that are not observed in wild-type flies [5,156,158].

Inhibiting apoptosis in neuroblasts compared to neurons should have a larger effect given that they continue to proliferate. However, this does not seem to affect the nervous system massively. Eliminating *rpr* and *grim* in the abdominal neuromeres leads to a block in neuroblast PCD, which in turn gives rise to a large number of neurons that are incorporated seemingly normally into the abdominal adult nervous system [33]. It is interesting to note that the fly hatches with an enlarged abdominal nervous system, which seems to introduce a physical constrain for copulation [184].

There are several questions that remain unanswered. Although 'undead' supernumerary neurons and glia seem to incorporate relatively normally in the nervous system, it has not been assessed how this affects circuit

function and behavior. Moreover, although 'undead' neuroblasts initially continue to proliferate, they have not been reported to persist to adulthood. There have been reports of caspase-independent cell engulfment, which is not affected in H99 mutants or p35-expressing cells and recapitulates the cell death pattern [146].

Cell cycle exit or apoptosis

Under wild-type conditions, the life of a neuroblast ends by one of two mechanisms: the neuroblast either divides terminally to give rise to two neurons or glia, or it undergoes apoptosis. What is the functional significance of the two mechanisms? Do they represent different solutions to the same problem (elimination of the neuroblast) or do they lead to different end products? One possibility is that neuroblasts undergoing apoptosis produce a signal necessary for neuronal maturation or axonal targeting, in a manner similar to what is observed in imaginal disk cells, where Wg and other molecules induce compensatory proliferation when undergoing apoptosis [185,186].

Embryos mutant for *prospero*, a gene that promotes cell cycle exit, display an increase in *reaper*-mediated apoptosis [187]. Conversely, in embryonic abdominal neuroblasts that are eliminated by PCD, the time that neuroblasts proliferate is expanded in *prospero* mutants, indicating a potential cell cycle exit before PCD occurs [118,187]. It is therefore possible that both mechanisms are employed in order for the animal to avoid overproliferating (oncogenic) cells. As already discussed, mushroom body neuroblasts have a fail-safe mechanism that assures neuroblast removal in the absence of RHG activity.

When a neuroblast undergoes apoptosis, its proliferative capacity is lost. On the other hand, neuroblasts that terminally divide may be able to pass this capacity to their progeny, leaving open the opportunity for later cell divisions. The terminal division of neuroblasts often gives rises to two glial cells, such as, for example, in the OPC of the developing optic lobe [95]. The same has been observed in vertebrate systems; for example, Müller glia are generated in the latest temporal window by the retinal progenitor cells after the production of retinal ganglion cells, interneurons, cones, and rods [188]. Moreover, Müller glial cells have been shown to retain proliferative capacity and to be able to divide and produce both Müller glial cells and other retinal neurons [189–191]. Therefore, it is possible that in the case of neuroblast terminal division, the capacity to proliferate is transferred by the neuroblast to one (or both) of its two progeny, which are usually glial cells. In favor of this argument, glial cells in multiple contexts have been shown to maintain mitotic activity [192]. This capacity may reveal itself immediately by the amplification of the glial population, or later upon activation in adulthood [193–195]. This difference in proliferative capacity may be mediated by the different levels of Prospero inherited by the terminally dividing neuroblast to its progeny. Prospero has been shown to influence proliferative capacity according to its expression levels; absence of Prospero leads to proliferation, low levels of Prospero induce quiescence, while high levels of Prospero promote differentiation [196].

Conclusion

The *Drosophila* nervous system has been an instrumental model system for understanding the mechanisms of programmed cell death, as well as its developmental significance. With the complete identification of all fly neuroblasts, and many of their lineages, a comprehensive comparison between homologous lineages will lead to a thorough understanding of how multiple developmental cues are integrated for the regulation of PCD. Finally, this knowledge lays the foundation for studying apoptosis during neural development in other insects and arthropods. This may eventually allow us to understand how and under which selective pressure the occurrences and mechanisms for regulation of PCD evolved over time.

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Author contributions

All authors contributed intellectually to this work. FPT and NK wrote the manuscript.

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