Programmed cell death in the embryonic central nervous system of Drosophila melanogaster

Ana Rogulja-Ortmann, Karin Lüer, Janina Seibert, Christof Rickert and Gerhard M. Technau*

Although programmed cell death (PCD) plays a crucial role throughout Drosophila CNS development, its pattern and incidence remain largely uninvestigated. We provide here a detailed analysis of the occurrence of PCD in the embryonic ventral nerve cord (VNC). We traced the spatio-temporal pattern of PCD and compared the appearance of, and total cell numbers in, thoracic and abdominal neuromeres of wild-type and PCD-deficient H99 mutant embryos. Furthermore, we have examined the clonal origin and fate of superfluous cells in H99 mutants by Dil labeling almost all neuroblasts, with special attention to segment-specific differences within the individually identified neuroblast lineages. Our data reveal that although PCD-deficient mutants appear morphologically well-structured, there is significant hyperplasia in the VNC. The majority of neuroblast lineages comprise superfluous cells, and a specific set of these lineages shows segment-specific characteristics. The superfluous cells can be specified as neurons with extended wild-type-like or abnormal axonal projections, but not as glia. The lineage data also provide indications towards the identities of neuroblasts that normally die in the late embryo and of those that become postembryonic and resume proliferation in the larva. Using cell-specific markers we were able to precisely identify some of the progeny cells, including the GW neuron, the U motoneurons and one of the RP motoneurons, all of which undergo segment-specific cell death. The data obtained in this analysis form the basis for further investigations into the mechanisms involved in the regulation of PCD and its role in segmental patterning in the embryonic CNS.

KEY WORDS: CNS, Programmed cell death, Segmental patterning, Neuroblasts, Lineages, H99, Drosophila

INTRODUCTION

Programmed cell death (PCD) is a prominent feature of development in all metazoan organisms, functioning in the control of cell numbers, in the removal of redundant structures and in the elimination of misspecified or harmful cells (reviewed by Jacobson et al., 1997). Selective cell death also provides an elegant mechanism for spatial patterning of developing tissues and organs (reviewed by Rusconi et al., 2000), and is employed in refining the nervous system in both vertebrates and invertebrates (reviewed by Oppenheim, 1991; Truman et al., 1992), in digit formation in higher vertebrates, in removing larval tissues during insect morphogenesis and in a number of other developmental processes. In these processes, tight spatio-temporal regulation of PCD is required to ensure precise elimination of redundant cells. How this regulation is achieved, and how PCD is integrated with cell determination and differentiation during development, are fundamental questions in developmental biology that remain largely unanswered.

The fruitfly Drosophila melanogaster renders itself useful to investigations of PCD, as the genes required for the initiation and execution of PCD have been cloned and there are numerous genetic tools available to allow manipulation of genes and developmental processes. Our goal has been to investigate mechanisms involved in the regulation of PCD in the developing CNS of the Drosophila embryo, particularly with respect to its role in segmental patterning. We provide a basis for these investigations by pursuing a more detailed analysis of cell death in the embryonic CNS, and by establishing single-cell models for further examination of mechanisms regulating developmental cell death.

In Drosophila, significant amounts of apoptotic cells have been observed in the embryonic CNS from the early stages of CNS formation to the end of embryogenesis (Abrams et al., 1993). Several studies over the last decade have identified different kinds of apoptotic cells in the CNS (White et al., 1994; Sonnenfeld and Jacobs, 1995; Hidalgo et al., 2001; Peterson et al., 2002; Lundell et al., 2003; Miguel-Aliaga and Thor, 2004; Karcavich and Doe, 2005). In most cases, the developmental signals responsible for inducing PCD in these cells are unclear. Embryonic neuroblast (NB) PCD has been shown to require the prosapoptotic gene reaper (rpr) (Peterson et al., 2002), but it is not known how rpr is activated to induce PCD in these NBs. In the third-instar larva, a pulse of the Hox protein Abd-B induces PCD in the dividing abdominal postembryonic NBs through activation of one or more of the three prosapoptotic genes Hid (Wrinkled – Flybase), rpr and grim, and thus limits the production of neural cells in the abdominal CNS (Bello et al., 2003). Whether a similar signal is involved in the death of the embryonic NBs remains to be investigated. Other groups have reported PCD occurring in postmitotic differentiated neural cells. Sonnenfeld and Jacobs (Sonnenfeld and Jacobs, 1995) were the first to report degeneration of differentiated midline glial cells upon completing their function in the morphogenesis of commissural axon tracts in early embryogenesis. Hidalgo and colleagues (Hidalgo et al., 2001) showed that survival of longitudinal glia (LG) depends on the Neuregulin trophic factor homolog Vein. Miguel-Aliaga and Thor (Miguel-Aliaga and Thor, 2004) found that the pioneer neurons dMP2 and MP1 undergo segment-specific PCD at the end of embryogenesis, and that the Hox gene Abd-B is required for the survival of these cells in posterior segments of the ventral nerve cord (VNC). Several studies (Novotny et al., 2002; Lundell et al., 2003; Karcavich and Doe, 2005) have reported apoptosis among the progeny of the neuroblast NB7-3. One to two of the six postmitotic cells produced in this lineage undergo apoptosis and these are the first reported examples of the death of
clearly identifiable, undifferentiated cells in the *Drosophila* embryonic CNS. Furthermore, Notch was identified as the apoptotic signal in the NB7-3 lineage (Lundell et al., 2003), but exactly how it activates the apoptotic pathway is unknown.

Despite the obvious importance of PCD in *Drosophila* development, only a very general overview of the occurrence of PCD in the developing embryonic CNS has been provided to date (Abrams et al., 1993). A systematic analysis of the number, segmental pattern and identity of dying cells has not been made. Such a detailed analysis would provide an important foundation for further research on mechanisms regulating developmental cell death. We present here the results of three approaches taken to gain insight into the occurrence and role of PCD in the embryonic CNS of *Drosophila melanogaster*: (1) tracing the spatio-temporal pattern of apoptotic cells in the developing wild-type CNS, as well as comparing the total cell numbers in thoracic and abdominal neuromeres of wild-type and PCD-deficient embryos; (2) examining the clonal origin, development and axonal projection patterns of additional cells in PCD-deficient embryos by Dil labeling of NB lineages; and (3) analysis of specific cell subpopulations in PCD-deficient embryos using various cell markers, and determination of the timing of PCD and the identity of some of these cells in the wild type, in order to establish models for studying mechanisms of PCD regulation.

**MATERIALS AND METHODS**

*Drosophila* stocks

Oregon-R was used as the wild-type strain. *Df(3L)H99/TM3, Sb* flies were obtained from the Bloomington *Drosophila* Stock Center and rebalanced over *TM6b, abdA-lacZ and TM6, ubi-GFP* balancer chromosomes.

**Immunohistochemistry**

Following dechorionization in 7.5% bleach, embryos from overnight collections were deproteinized and fixed in 4% formaldehyde in PEMS buffer (0.1M Pipes, 1mM MgSO4, 1mM EGTA, 1.2 M Sorbitol, all Sigma) for 25 minutes. The fixed embryos were dehydrated by a 10 minute wash in methanol. For staining with dianemobenzidine (DAB, Sigma), embryos were incubated in 3% H2O2 solution in ethanol for 15 minutes. Primary antibodies used were mouse anti-BP102 (1:20), mouse anti-FasII (1:10), mouse anti-Engrailed/Invected (1:2) and mouse anti-Even-skipped (1:2), all from Developmental Studies Hybridoma Bank; mouse anti-BrdU (1:3.5, Becton-Dickinson), rabbit anti-human activated caspase-3 (1:50, Cell Signalling Technology), rat anti-Gooseberry distal (1:2, R. Holmgren, Northwestern University, Evanston, IL, USA), rat anti-Gooseberry proximal (1:2, R. Holmgren), guinea pig anti-anti-Hb (1:1000, J. Skeatham, Washington University School of Medicine, St. Louis, MO, USA), rabbit anti-Robo (1:500) (Halter et al., 1995), mouse anti-Ladybird early (1:2, K. Jagla, Institut National de la Santé et de la Recherche Médicale, Clermont-Ferrand, France), rabbit anti-Eagle (1:500) (Dittrich et al., 1997), mouse anti-Eagle (1:10, C. Doe, University of Oregon, Eugene, OR, USA), rabbit anti-Even-skipped (1:1000, M. Frasch, Mount Sinai School of Medicine, New York, NY, USA), rabbit anti-B-gal (1:2000, Cappel). The secondary antibodies used were anti-mouse-biotin, anti-rat-biotin, anti-guinea pig-biotin, anti-rabbit-biotin, anti-mouse-FITC, anti-rat-FITC, anti-guinea pig-Cy5, anti-rat-Cy5, anti-rabbit-Cy5, anti-mouse-Cy3, anti-guinea pig-Cy3, anti-rabbit-Cy3 (1:250, all from donkey, all Jackson Immunoresearch Laboratories), anti-mouse-Cy5 from goat (1:250, Jackson Immunoresearch Laboratories) and donkey anti-mouse-Alexa488 (1:250, Molecular Probes). For DAB stainings, the ABC Kit from Vectastain was used. Color images were produced using a Zeiss Axioplan 2 microscope. The Leica TCS SPI confocal microscope was used for fluorescent imaging, and the images were processed using Leica Confocal software and Adobe Photoshop.

**Cell counts**

Embryos were fixed as described above, then incubated for 40 minutes in a 2 µg/ml RNase solution. Following washes in PB and PBS, embryos were embedded in 70% glycerol. Fillet preparations were made and stacks recorded with Nomarski optics. Sections were taken every 0.98 µm, using a Zeiss Axiostar 2 microscope equipped with a motorized stage. Cells were counted in one hemineuromere of segments T2 and T3, and from A3 to A5. To this purpose, cells in each section of the stack were marked using Adobe Photoshop. To avoid marking cells that had been marked in a previous section of the stack, subsequent sections were projected on top of each other and compared. The marked cells counted in each section were added to give the sum of all cells in one stack.

**BrdU labeling**

BrdU (Sigma) was injected as previously described (Prokop and Technau, 1991). Injected embryos were allowed to develop until stage 17 at which point filament preparations of the CNS were made and fixed in 18% formaldehyde for 2 minutes. After washing, the preparations were treated for 4 minutes with 2N HCl and blocked in 10% goat serum for 15 minutes. Antibody staining was performed as described above.

**Dil labeling**

Dil labeling was performed as previously described (Bossing et al., 1996). Embryos from the *Df(3L)H99/TM6, ubi-GFP* fly stock were labeled. Heterozygous embryos were used as controls, as their CNS lineages did not differ from the published description of the wild type (see Bossing et al., 1996; Schmidt et al., 1997). The *Df(3L)H99* homozygous embryos were distinguished on the basis of head involution and thicker midline phenotypes. Clones were imaged using the Zeiss Axioplan 2 microscope and the images processed as described above. For illustrations, a Zeiss Axioplan microscope with a Camera lucida was used and the drawings produced using Adobe Illustrator software.

**RESULTS**

We began our investigations by comparing the CNS morphology and total CNS cell number in wild-type (wt) embryos with PCD-deficient embryos homozygous for the deficiency *Df(3L)H99 (H99)* (White et al., 1994). One typical feature of *H99* embryos is a variably penetrant defect in head involution, visible from stage 15 to the end of embryogenesis (Abbot and Lengyel, 1991). In other tissues, these embryos show a range of phenotypes from problems with germ-band retraction (the earliest phenotype we were able to observe) and defects in gut development and CNS condensation, to those with hardly any macroscopically visible phenotype. In order to maximize our chances of observing differences between the wt and *H99* CNS, we analyzed embryos at late developmental stages (late 16 or early 17), and we considered cases both with and without defects in CNS condensation, as this did not seem to affect our observations.

**Comparison of morphology and cell number in the CNS of wt and *H99* embryos**

As a consequence of the lack of PCD, the CNS in late *H99* embryos is wider than in wt, but it has a fairly normal appearance. The midline is widened and disrupted due to the survival of several midline glia (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995). As has been reported previously (Zhou et al., 1995; Dong and Jacobs, 1997), the commissures and the longitudinal connectives, visualized by BP102 staining, are broadened and the junctions between them thickened due to additional axons, but their pattern is not changed (Fig. 1A,B). This indicates that at least some of the supernumerary neurons differentiate and extend axonal projections within the normal commissures and longitudinal connectives. In general, the axons seem to find and follow their normal pathways in *H99* embryos, as there was no obvious phenotype in the FasII pattern (Fig. 1C,D). The three longitudinal fascicles formed and, apart from a variably ‘bumpy’ appearance, looked similar to wt. The peripheral transverse, segmental and intersegmental nerves appeared normal, as well as the four nerve branches (SNa-d) (data not shown).
nerves all appeared to be of normal thickness and it was difficult to tell whether they contained supernumerary axons. The glia pattern, apart from a moderate misplacement of some cells, was also surprisingly normal, both in the VNC (Fig. 1E,F) and in the periphery (data not shown). Also, as explained in more detail below, the number of Repo-positive glial cells was unaltered in H99 embryos. We conclude that the CNS structure in late stage 17 embryos is generally wider than that of wt due to additional cells. The positioning of glia is only slightly altered pattern. The H99 embryo shown in D has a more extreme phenotype in that the fascicles are somewhat disordered and fuzzy. (E,F) Gial cells, visualized with the anti-Repo antibody. A stack of scans was made throughout the CNS and the scans then projected together to show all Repo-positive cells. The positioning of glia is only slightly affected in H99. All images show four abdominal segments (A3 to A6) of late stage 16 embryos; anterior is up.

Fig. 1. The CNS of homozygous H99 embryos is not grossly deformed. (A,B). Axon tracts are visualized using the BP102 antibody in wt (A) and H99 (B) embryos. Their pattern is similar, although thickened junctions between the longitudinal connectives and the commissures (arrow), and a widened midline (bracket) are visible in H99. Note that the CNS of H99 embryos is generally wider than that of wt due to additional cells. (C,D) Fast staining of axons reveals a variably altered pattern. The H99 embryo shown in D has a more extreme phenotype in that the fascicles are somewhat disordered and fuzzy. (E,F) Gial cells, visualized with the anti-Repo antibody. A stack of scans was made throughout the CNS and the scans then projected together to show all Repo-positive cells. The positioning of glia is only slightly affected in H99. All images show four abdominal segments (A3 to A6) of late stage 16 embryos; anterior is up.

numbers per thoracic hemisegment (hs) (524 ± 34, n=10) and per abdominal hs (451 ± 36, n=12) were slightly higher than in wt. Despite the greater variability in the H99 strain, the difference between the wt and H99 abdominal cell counts is statistically significant (P<0.001). In contrast to wt, cell numbers in H99 did not significantly change from stage 16 (see above) to 17: 534 ± 83 (n=10) in thoracic neuromeres and 462 ± 80 (n=18) in abdominal neuromeres. At stage 17, we observed considerable differences between wt and H99 embryos in both thoracic [354 ± 58 (n=8) and 534 ± 83 (n=10), respectively] and abdominal segments [286 ± 34 (n=12) and 462 ± 80 (n=18), respectively] (Fig. 2). The difference is statistically significant in both cases (P<0.001).

The supernumerary cells in the CNS of H99 embryos are most likely to be cells whose fate in wt would be PCD. It is, however, also conceivable that at least some of these cells derive from the continuing division of NBs which would normally undergo PCD after they have generated their progeny, or from the division of ganglion mother cells (GMCs) that are born, but normally undergo apoptosis without dividing. To test this, we performed BrdU labeling experiments. It has been shown that in the wt VNC, there are few divisions taking place after stage 16 (Prokop and Technau, 1991). In H99 embryos injected with BrdU at early stage 16 and fixed at late stage 17, there were only a few more labeled cells than in wt embryos injected at the same stage (data not shown). When BrdU was injected at early stage 17, we found two classes of H99 embryos at late stage 17: those that differed little from wt (Fig. 3, compare A,B), and those with a greater number of labeled cells than in the wt (Fig. 3, compare A,C). These results show that some of the supernumerary cells in H99 embryos are cells that are never born in the wt CNS. Also, the variability in the BrdU uptake within the H99 mutant strain appears to be a consequence of surviving NBs or GMCs either not dividing at all, or going through a variable number of divisions. We also observed a difference in the amounts of BrdU-labeled cells between the thorax and abdomen of H99 embryos injected at early stage 17 (Fig. 3B,C). In the thoracic region, the

![Cell death in the embryonic CNS of Drosophila](image-url)
majority of BrdU-labeled cells were lateral, whereas in the abdomen the labeled cells were distributed more equally between the lateral and medial regions of each segment. There were fewer cells stained in the thoracic region in general, which is in agreement with the previously published observation that the majority of abdominal NBs undergo PCD, whereas the thoracic ones mostly enter quiescence at the end of embryogenesis (Truman and Bate, 1988).

**Temporal profile of cell death throughout embryonic CNS development**

In order to examine the occurrence of PCD in the developing nervous system of the *Drosophila* embryo, we made use of a polyclonal antibody raised against the activated form of the human caspase-3 protein. This antibody has previously been shown to recognize apoptotic cells in *Drosophila* tissues (Brennecke et al., 2003), and it does not show any staining in homozygous *H99* embryos (data not shown). In the CNS of wt embryos, PCD first appeared around the beginning of stage 11. PCD then continued to occur until the end of embryonic development (Fig. 4) (see also Abrams et al., 1993). The number of dying cells per abdominal hemisegment increased steadily from stage 11 to reach a peak in mid-development (stage 14), at about 20 cells/h (n=33). After this point, the number of dying cells stayed more or less constant until the end of embryonic development. As has been reported previously (Abrams et al., 1993), the spatial distribution of activated Caspase-3-positive cells at any stage shows both a regular, segmentally repetitive distribution, as well as a random one (data not shown).

Fig. 3. BrdU staining in late stage 17 wt and *H99* embryos. All embryos were injected at early stage 17. (A) Wt VNC. Sporadic staining can be seen in the abdominal segments. In the thoracic region, BrdU-labeled cells are found only in the very lateral region of each hemisegment. (B, C) VNCs of two *H99* embryos, representing the two classes of staining found in *H99* mutants. One shows a similar amount of staining as in wt (B), whereas the other has many more BrdU-positive cells (C). In both cases, there is a concentration of dividing cells in the lateral region of each abdominal hemisegment. The thoracic segments in B and C show reduced staining in the medial regions. Anterior is to the left in all images.

**Fig. 4. Profile of Caspase-dependent PCD in the embryonic CNS.**

Activated-Caspase-3-positive cells in the wt embryonic CNS were counted in abdominal hemisegments over the course of development. PCD in the CNS begins at stage 11 and increases until stage 14. The levels remain high until the end of embryonic development. Bars represent s.d.; n=36-51 (for all stages). Developmental stages are indicated above the bars.

**Dil labeling of neuroblast lineages in *H99* embryos**

We next investigated the development of individual NB lineages in embryos that lack PCD, with a view to determining how many additional cells, if any, each NB lineage would make, how the additional cells develop (e.g. whether they differentiate, migrate, extend axons, etc.), and how their potential axons project. We performed labeling experiments with the cell lineage tracer Dil (Bossing and Technau, 1994) in homozygous *H99* embryos (preferentially in the abdomen). We obtained clones for almost all 30 NBs, analyzed their cell number and axonal projections and compared them with the published descriptions of wild-type clones (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). The results are summarized in Table 1 (for selected images see Figs 5, 6, and Figs S1, S2 in the supplementary material). The clones were sorted into four groups on the basis of their appearance: (1) clones showing no difference in cell number or morphology as compared with wt; (2) clones with additional cells and wild-type-like axonal projections; (3) clones with additional cells and axonal projections different from those in wt counterparts; and (4) clones showing no tagma-specific phenotype in wt, but differing between abdomen and thorax in *H99*. The groups are described and the most interesting examples of clones are shown below.

1. **Clones with no additional cells**

Abdominal NB1-1a was the only NB lineage which we repeatedly (n=5) found to be unchanged in *H99* embryos (two clones of 10 cells and three clones of 11 cells) (Fig. 5A-C, Table 1). The wt consists of 9-11 cells (three subperineurial glial cells, the aCC motoneuron, the pCC interneuron, and a cluster of 4-6 interneurons) (Udolph et al., 1993). In only one out of five cases in *H99* did we observe an axon which branched out of the typical axon bundle (data not shown); however, the clone contained a wild-type number of cells (11) and we therefore assume that the axon had been misrouted. We observed two other lineages (thoracic NB1-3t and abdominal NB6-4a) in which the *H99* embryos did not seem to differ from their wt counterparts (Table 1 and data not shown); however, due to a low sample number (n=1), we consider these observations inconclusive.
Table 1. Comparison of Dil-labeled clone sizes and projections between wt and H99 embryos

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<th>Cell # (H99)</th>
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*a Candidate abdominal pNBs.

† Candidate thoracic NBs that undergo apoptosis.

‡ The group that each NB belongs to is indicated as follows: 1, Clones with no additional cells; 2, Clones with additional cells and wild-type-like axonal projections; 3, Clones with additional cells and atypical axonal projections; 4, Clones whose phenotypes differ between abdomen and thorax. a, abdominal clones; t, thoracic clones.

2. Clones with additional cells and wild-type-like axonal projections

For 14 NB lineages we obtained clones in H99 embryos which clearly and repeatedly contained more cells than their wt counterparts but showed a wild-type-like projection pattern. These were NB2-1, NB2-2a, NB2-4a, NB2-5, NB3-1a, NB3-2, NB3-5, NB4-4, NB5-1, NB5-4a, NB5-5, NB6-1, NB7-1 and NB7-3 (for details see Table 1). All of these clones were easily identifiable on the basis of their axonal projections (for selected clones, see Figs S1, S2 in the supplementary material). Due to the lack of information on axon numbers per fascicle in wt clones, and because of tight packaging of axons in the bundles, we were generally not able to determine whether the projections in H99 contained additional axons or not. The only exception was NB7-3, which contains only four cells in the wt: three contralaterally projecting interneurons (EW1-3) and one ipsilaterally projecting motoneuron (GW) (Fig. 5D) (Higashijima et al., 1996; Bossing et al., 1996; Dittrich et al., 1997; Schmid et al., 1999; Novotny et al., 2002). We obtained five examples of this clone in H99 embryos, comprising 9-10 cells (Fig. 5E,F). Although their projections followed the wt pattern, we were able to identify an additional motoprojection in all five cases. Regarding the interneuronal projections, it was not possible to determine the number of axons they contained, as these are bound together too tightly.

We found four further NBs to have larger clones in H99 than in wt (NB2-2t, NB4-1, NB5-6a and NB6-4t) (Table 1 and data not shown) but as we only obtained one clone for each of these, we can draw no solid conclusion about PCD in these lineages.

3. Clones with additional cells and atypical axonal projections

We obtained clones of four NB lineages (NB4-2, NB5-3, NB7-2 and NB7-4) in H99 embryos which showed additional cells and axonal projections that have not been observed in their wt counterparts.

NB4-2 contains 10-16 cells (7-13 interneurons, 3 motoneurons) in wt (Bossing et al., 1996) (Fig. 6A). We obtained three clones of this lineage in H99, one thoracic containing 16 cells, and two abdominal with 17 and 25 cells (Table 1, Fig. 6B,C). One of the abdominal clones exhibited a wild-type-like projection pattern, whereas the other abdominal and the thoracic clone contained two additional motoneurons each, whose axons project ipsilaterally in the anterior direction. This lineage can also be placed in group 4, and as such is mentioned again below.

NB5-3 is another example of a lineage with additional cells and projections in H99 (Table 1, Fig. 6D-F). In wt this lineage contains 9-15 cells. These are mostly interneurons, except for one motoneuron in the thoracic and first abdominal segments (Fig. 6D) (Schmid et al., 1999). The cells are arranged in two clusters, one lying medially and projecting across the anterior commissure, and the other lying laterally and projecting through the posterior commissure (Bossing et al., 1996; Schmid et al., 1997; Schmid et al., 1999). We obtained seven abdominal clones in H99 embryos, containing 19-27 neurons. In two of these clones, we found at least one additional ipsilateral axon projecting anteriorly (Fig. 6E,F), and in four further clones we identified structures that resembled the beginnings of axons growing out in the same direction. In addition, all seven clones contained a motoneuron. As the labeled clones were found in various abdominal segments (A1, A2, A3, A6 and A7), we conclude that in the wt the motoneuron is born in all segments and undergoes PCD (most likely before growing an axon) in A2 to A8, thus representing an example of segment-specific cell death.

The NB7-2 lineage normally consists of 8-14 interneurons (mostly 12), whose projections form two fascicles. One traverses contralaterally across the posterior commissure (7-2c) and the other extends ipsilaterally to the posterior (7-2l) (Fig. 6G) (Bossing et al., 1996). In H99, we obtained two clones, with 21 and 28 neurons, that project an additional axon contralaterally through the posterior commissure, alongside the wild-type-like fascicle (Table 1, Fig.
6H, I). We believe this to be a separate axon, and not a case of loose fasciculation, because the position of the axon was exactly the same in both clone examples, i.e. it comes from the cells lying laterally within the clone. In a loose fascicle, one would expect the axons to be positioned more variably and fairly close together.

The NB7-4 lineage contains 8-12 interneurons and 5-7 glial cells in the wt. The interneurons project contraterally across the posterior commissure (Fig. 6I) (Schmidt et al., 1997). In H99, this lineage contained 18-24 neurons and 4-7 glia. Additional axons projected contraterally through the anterior commissure of the next segment in all clones obtained (six abdominal and one thoracic, Fig. 6K,L).

NB5-4t also exhibited atypical projections in H99 (see Fig. S2 in the supplementary material); however, as we obtained only one clone we cannot draw firm conclusions about this lineage.

4. Clones whose phenotypes differ between abdomen and thorax

In six cases we obtained NB lineages which seemed differently affected in the abdomen and thorax of H99 embryos. These were NB4-3, NB3-3, NB4-2, NB5-2, NB6-2 and NB1-2. In wt, NB4-3 consists in both tagmas of 8-13 motoneurons whose projections all leave the CNS through the segmental nerve (Schmidt et al., 1997). This lineage often comprises an epidermal and a sensory subclone, which we have also observed in two out of five cases in H99 embryos. We reasoned that any cell which is determined to express one of these markers, but undergoes cell death at some point in development, would be likely to continue to express this marker if PCD is prevented. In fact, this has been shown for apoptotic midline glia (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995) and other apoptotic cells in the CNS (Novotny et al., 2002; Miguel-Aliaga and Thor, 2004).

In H99 embryos, we therefore expected to see all the additional cells which continued to express a particular marker. In parallel, we examined the overlap between the activation of Caspase-3 and individual marker expression in various developmental stages of wt and homozygous H99 embryos. We reasoned that any cell which continued to express a particular marker, would be likely to continue to express this marker if PCD is prevented. In fact, this has been shown for apoptotic midline glia (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995) and other apoptotic cells in the CNS (Novotny et al., 2002; Miguel-Aliaga and Thor, 2004).

Identification of dying cells

We next attempted to identify the dying cells in the CNS more closely. To do this, we selected a number of molecular markers that are known to be expressed in smaller or larger groups of cells in the VNC [e.g. Repo, dHb9 (Exex – Flybase), Eve], and compared the extent of their expression in late developmental stages of wt and homozygous H99 embryos. We reasoned that any cell which is determined to express one of these markers, but undergoes cell death at some point in development, would be likely to continue to express this marker if PCD is prevented. In fact, this has been shown for apoptotic midline glia (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995) and other apoptotic cells in the CNS (Novotny et al., 2002; Miguel-Aliaga and Thor, 2004).

In H99 embryos, we therefore expected to see all the additional cells which continued to express a particular marker. In parallel, we examined the overlap between the activation of Caspase-3 and individual marker expression in various developmental stages of wt embryos, in order to determine the time of death for some of these cells. We chose embryos in mid-development (stages 13 and 14) as our analysis of cell death distribution indicated that it is most frequent in these stages. We also examined embryos in a late developmental stage (late stage 16) to identify cells that are removed towards the end of embryogenesis.

The glial marker Repo did not show any obvious difference in the extent of expression between wt and H99 embryos (see Fig. 1E,F). We therefore performed precise cell counts for Repo-expressing cells at late stage 16, including glia in the CNS and the peripheral glia that are born in the CNS and then migrate out along the nerves. In wt embryos, a total of 34.17±0.65 cells/hs were counted (n=30), and in H99 we found 34.77±0.73 cells/hs (n=30). We conclude that the number of glial cells is not significantly changed in H99 embryos, and that the great majority of dying cells in the embryonic CNS are neurons or undifferentiated cells.
As anticipated, markers expressed in large groups of cells, such as dHb9, Gooseberry and Engrailed (Fig. 7A,B and data not shown), stained more cells in H99 than in wt. Most of these markers also showed, at least in some of the developmental stages examined, overlap with activated Caspase-3 staining in a few cells in wt embryos (Fig. 7C and data not shown). In most cases, we were not able to identify these cells due to the extent of marker expression. Closer identification was possible only for cells expressing dHb9, a homeodomain protein expressed in a specific subset of neurons (Broihier and Skeath, 2002). At stage
14, for example, we found cells that most likely correspond to one of the RP motoneurons (RP 1, 3, 4 or 5), co-labeled with activated Caspase-3. This cell death is specific to segments A7 and A8, and activated Caspase-3 staining of this cell was detected at this stage in 27.3% of hemisegments analyzed (n=22; Fig. 7C).

Some molecular markers which are expressed in small subsets of cells in the VNC showed, as has already been reported (De Graeve et al., 2004; Novotny et al., 2002), interesting pattern changes in H99 embryos (Fig. 8). Ladybird early (Lbe) is a homeobox transcription factor involved in several developmental processes (Jagla et al., 1994; Jagla et al., 1997; Jagla et al., 1998).

In the embryonic CNS it is required for the correct development of glial cells derived from NB5-6 and of neurons derived from NB5-3 (De Graeve et al., 2004). De Graeve et al. also showed that late H99 embryos have additional Lbe-positive neuronal cells in the VNC. We made the same observation, and determined that these Lbe-positive cells die in stages 13 and 14 (Fig. 8A-C). As for the dHb9 marker, this suggests that these cells die prior to or at an early stage of differentiation. The experiments we are currently undertaking should provide us with the possibility to precisely identify each of these cells based on the combination of markers it expresses. Another marker we used, the zinc-finger protein Eagle (Eg), has been described as being required for proper differentiation of cells in four NB lineages, NB2-4, NB3-3, NB6-4 and NB7-3 (Hiashi-Hijima et al., 1996; Dittrich et al., 1997). We focused our analysis on the NB7-3 lineage, which consists of only four neurons: three interneurons and one motoneuron. Previously published data show that PCD is involved in the formation of this lineage, as late H99 embryos have additional cells in the NB7-3 lineage (Novotny et al., 2002). It has been shown that these cells are not fully differentiated, and that the death of some of these cells depends on Notch activity (Lundell et al., 2003). Our results are in agreement with the published data (Fig. 8D,E), although we also observed apoptotic Eg-positive cells at stage 13 (not shown) and at late stage 16 (Fig. 8F). We identified the cell undergoing PCD at late stage 16 as the GW motoneuron, and determined that it is removed specifically in segments T3 to A8 in 38.9% of cases (n=108). We thus showed that differentiated NB7-3 progeny can also undergo PCD. In T1 and T2 this cell survives into larval stages. Even-skipped (Eve), another marker we used, is a homeodomain transcription factor expressed in a very restricted and well-described pattern in the embryonic CNS (Frasch et al., 1987; Doe et al., 1988). Comparing the Eve expression pattern in anterior abdominal segments at late stage 16, we did not see any difference between wt and H99 embryos. However, some or all of the U neurons in segments A6 to A8 of wt embryos were missing, whereas they were still present in homozygous H99 embryos (Fig. 8G,H). These motoneurons, belonging to the NB7-1 lineage, underwent PCD in stages 14 and 15 as seen by Caspase-3 activation (Fig. 8I).

Further experiments are underway to investigate possible factors that regulate PCD of these cells.

**DISCUSSION**

Programmed cell death is an integral part of animal development, and as such is also involved in spatial patterning of tissues and organs. In the *Drosophila* embryonic CNS, factors regulating this developmental cell death have just begun to be identified. In order to establish a basis for further investigations into these mechanisms, we set out to analyze the distribution of PCD throughout CNS development, and to identify apoptotic cells in order to use them as models for these investigations.
The CNS of PCD-deficient embryos is not drastically affected at the macroscopic level

In our analysis of PCD distribution we found that, macroscopically, the CNS of wt and PCD-deficient (H99) embryos do not show large differences. Our observations indicate that the supernumerary cells do not disturb developmental events in the CNS of H99 embryos, such as cell migration and axonal pathfinding. The glial cells mostly find their appropriate positions accurately. The DiI-labeled NB lineages were, in the majority of cases, easily identifiable based on their shape, position and axonal pattern, despite the supernumerary cells. The FasII pattern showed that the axonal projections form and extend along their usual paths. In fact, the supernumerary cells themselves are capable of differentiating i.e. expressing marker genes and extending axons, as shown by clones of several NBs and by cell marker expression analysis in H99 (e.g. NB7-3).

Pattern and degree of cell death in the ventral nerve cord

It has been shown that a large number of CNS cells undergo PCD during embryonic development (Abrams et al., 1993). The distribution of activated Caspase-3-positive cells in wt embryos suggests that the death of some cells is under tight spatial and temporal control, as revealed by their regular, segmentally repeated occurrence. Other dying cells were rather randomly distributed, suggesting a certain amount of developmental plasticity. The overall counts of Caspase-3-positive cells give an estimate of the numbers of dying cells at a given time. They indicate that PCD becomes evident in the CNS at stage 11 and is most abundant in the late embryo (from stage 14). It is however difficult to estimate the total number of apoptotic cells throughout CNS development by anti-Caspase-3 labeling, because the cell corpses are removed fairly quickly. We therefore counted the total number of cells per thoracic and abdominal hemineuromere in the late embryo. Comparison between stage 16 and stage 17 wt embryos indicates that 25-30 % of all cells are removed in both tagmata after stage 16, which in turn suggests that the total percentage of removed cells must be high, as PCD occurs at high levels already from stage 14 on. In comparison to the developing nervous system of C. elegans, where PCD removes about 10% of cells, and of mammals, where this number can be as high as 50-90%, PCD in the fly CNS appears to show an intermediate prevalence. This lends support to the hypothesis of an increasing contribution of PCD in shaping more advanced nervous systems during evolution.

Comparisons between wt and H99 reveal, as expected, a greater number of cells in both tagmata of H99 embryos (151 % increase in the thorax and 162 % in the abdomen at stage 17). These additional cells in H99 may reflect the total number of cells normally undergoing cell death until stage 17. However, there is a large variability in the total number of cells, especially within the H99 strain. In wt embryos, it seems to be more pronounced in the thorax and at stage 17, which might be a consequence of variable amounts of PCD occurring until this stage. The even higher variability within the H99 strain (both in thorax and abdomen) is likely to reflect variable numbers of additional cell divisions. The great majority of abdominal NBs are normally removed by PCD after they have generated their embryonic progeny (Bray et al., 1989; White et al., 1994; Peterson et al., 2002), whereas in the thoracic neuromeres most of the NBs enter quiescence at the end of embryogenesis and continue dividing as postembryonic NBs in larval stages (Truman and Bate, 1988). Thus, there are few mitoses occurring in the wt CNS from stage 16 onwards (Prokop and Technau, 1991). Our BrdU labeling experiments revealed a high number of BrdU-positive cells in some H99 embryos injected at early stage 17. We assume that these are progeny of mitotic NBs and/or GMCs that survive and continue dividing, generating cells that do not exist in wt. Clones obtained by DiI labeling in H99 confirm this conclusion (see below). Our finding that surviving cells divide already in the embryo complement the results of Peterson et al. (Peterson et al., 2002), who found that, in reaper mutants, NBs in the abdominal neuromeres survive and generate progeny in larval stages.

Supernumerary cells can be specified as neurons but not as glia

Among the DiI-labeled clones in H99 embryos, we obtained very few NB lineages which did not differ from their wt counterparts. The majority contained, as expected, supernumerary cells. In some cases we could identify axons projected by these cells, which shows they are specified as neurons. In fact, in three cases (NB4-2, NB5-3 and NB7-3), we found these additional cells to be specified as motoneurons. As additional axons within a fascicle were generally difficult to identify, it is possible that these are not the only lineages which make additional motoneurons in H99. Whether these cells are normally born and apoptose, or originate from additional divisions of surviving NBs or GMCs, cannot be determined from these experiments, but similar observations have been made for both cases. Lundell et al. (Lundell et al., 2003) have shown that the normally apoptotic progeny of NB7-3 can express the neuronal differentiation markers Ddc and Corazonin when cell death is prevented. Also, the additional progeny of the surviving NBs in the reaper mutant larvae express the neuronal marker Elav, showing that cells which are never born in the wt are capable of becoming neurons (Peterson et al., 2002). It is interesting that none of these cells, regardless of their origin, are specified as glia. We did not observe any additional glia in the NB clones in H99 embryos, and we also found equal numbers of Repo-expressing glial cells in wt and H99. We conclude that PCD occurs almost exclusively in neurons and/or undifferentiated cells, and that lateral glia are not produced in excess numbers in the embryo. Furthermore, because it is likely that NBs, which normally die, stay in a late temporal window in H99, one could speculate that NBs in this window normally do not give rise to glia. Our results are not in agreement with the notion that LG are overproduced, and their numbers adjusted through axon contact (Hidalgo et al., 2001). Hidalgo et al. observe occasional apoptotic LG and it is possible that our method of counting does not allow a resolution fine enough to account for an occasional additional Repo-positive cell in H99 embryos. However, if LG were consistently overproduced, we would expect to observe a higher number of glia in H99 embryos. We assume that LG cell death may reflect a small variability in the number of cells needed, and not a general mechanism for adjusting glial cell numbers.

As already mentioned, we generally found no difference between Repo-expressing glia numbers in wt and H99. However, a small difference does become apparent when one separates the total cell counts into those in the CNS and those in the periphery: 25.67±0.45 cells/hs and 28.42±0.64 cells/hs for wt and H99, respectively, were counted in the CNS, whereas 8.50±0.28 cells/hs and 6.35±0.82 cells/hs for wt and H99, respectively, were found in the periphery. The reasons for this difference might be the greater width of the CNS in H99 embryos, and that the cues required for proper migration of the peripheral glia are disturbed by additional cells. Alternatively, the difference might be due to differentiation defects in these cells.
Atypical axonal projections in Dil-labeled H99 clones

In addition to NB clones with too many cells and wild-type-like axon projections in H99, we also obtained some lineages whose clones exhibited atypical projection patterns. We found these projections to belong both to motoneurons (e.g. NB4-2) and interneurons (e.g. NB5-3, NB7-2 and NB-7-4). NB4-2 normally produces two motoneurons (RP2 and 4-2Mar) and 8-14 interneurons (Bossing et al., 1996). In two out of three NB4-2 clones in H99 we found two additional motoneurons that project anteriorly, similar to RP2. One of the two clones was found in the thorax and had a normal cell number (16), whereas the other was abdominal and had too many cells (25). Thus, the two additional motoneurons are likely to be the progeny of divisions occurring in the wt, and not of an additional NB or GMC mitosis. The fact that the third NB4-2 clone (found in the abdomen and comprising 17 cells) did not show the same motoneuronal projections could be due to these cells not being differentiated at the time of fixation (we have occasionally observed clones of different ages in the same embryo), or they may not have differentiated at all. It would be interesting to determine the target(s) of these additional motoneurons and thereby perhaps gain insight into physiological reasons for their death. However, such an experiment has to await tools that allow us to specifically label the NB4-2 lineage, or these motoneurons, in the H99 mutant background.

The other three lineages (NB5-3, NB7-2 and NB7-4) all have atypical interneuronal projections. The cells which these atypical axons belong to may represent evolutionary remnants that are not needed in the Drosophila CNS. Alternatively, they might have a function earlier in development and be removed when this function is fulfilled. Such a role has been shown for the dMP2 and MP1 neurons, which are born in all segments and pioneer the longitudinal axon tracts. At the end of embryogenesis these neurons undergo PCD in all segments except A6 to A8, where their axons innervate the hindgut (Miguel-Aliaga and Thor, 2004). It is known that some cells of the NB5-3 lineage express the transcription factor Lbe, and that H99 mutants show about three additional Lbe-positive neurons per hemisegment, which mostly likely belong to NB5-3 (DeGraeve et al., 2003). Our Dil-labeling results complement this finding in that we also find four or more additional neurons in H99 clones. The supernumerary Lbe-positive neurons in H99 could possibly be the ones producing the atypical axonal projections.

Tagma-specific differences in H99 embryos

In the wt embryo, only eight NB lineages show obvious tagma-specific differences in cell number and composition (Bossing et al., 1996; Schmidt et al., 1997). Tagma-specific differences among serially homologous CNS lineages have previously been shown to be controlled by homeotic genes (e.g. Prokop et al., 1994a; Berger et al., 2005). Therefore, these lineages provide useful models for studying homeotic gene function on segment-specific PCD. In H99 embryos, we observed further lineages that were differently affected in the thorax and abdomen. These tagma-specific differences arise in a PCD-deficient background is an interesting question. For example, NB4-3 shows a wild-type cell number in the thorax (8 and 12-13), but has too many cells in the abdomen (15, 15 and 22). There are a couple of plausible scenarios to explain this observation. First, the development of the NB4-3 lineage, including the involvement of PCD, could actually differ in the thorax and abdomen of wt embryos, with the final cell number being similar by chance. The Dil-labeled clones allow determination of the final cell number, but do not reveal how this number is achieved. The difference would become obvious in an H99 mutant background, at least regarding the involvement of PCD. Second, and this possibility does not exclude the first one, the thoracic NB4-3 could become a postembryonic NB (pNB) and the abdominal NB4-3 might undergo PCD after generating the embryonic lineage. In H99, the abdominal NB would be capable of undergoing a variable number of additional divisions to generate a variable number of progeny. This would easily explain larger discrepancies in cell number between individual clones in H99 (e.g. the abdominal NB4-3 clone with 22 cells), and is in agreement with our occasional observations of H99 embryos with a very high CNS cell number per segment, and with the two observed classes of H99 embryos with high and low numbers of BrdU-positive cells.

NB6-2 is another lineage whose clones differ in the two tagmata of H99 embryos. In this case, the abdominal clones showed no difference to their wt counterparts, whereas the thoracic clones did (18 and 19 cells). Although no difference in cell number between thoracic and abdominal clones was reported for this lineage, a rather large count range (8-16 cells) was given (Bossing et al., 1996), which would allow for a thorax-specific PCD of two to three postmitotic progeny. Alternatively, the thoracic NB6-2 might undergo cell death upon generating its progeny, which would make it the first identified apoptotic NB in the thorax. When PCD is prevented, this NB may undergo a few additional rounds of division. The data obtained in our experiments do not counter this notion, but the number of clones obtained in the thorax was not sufficient to draw a definite conclusion. As the abdominal NB6-2 lineage in H99 did not differ from the one in wt, its NB may be one of the few abdominal postembryonic NBs (see below).

Identities of neuroblasts dying in the late embryo and of surviving neuroblasts resuming proliferation in the larva

As mentioned above, a specific set of NBs undergoes PCD in the late embryo, whereas surviving NBs resume proliferation in the larva as pNBs, after a period of mitotic quiescence (Bray et al., 1989; White et al., 1994; Peterson et al., 2002; Truman and Bate, 1988; Prokop and Technau, 1991; Prokop and Technau, 1994b). The identities of the individual NBs undergoing PCD versus those surviving as pNBs are still unknown. The sizes of NB lineages obtained in H99 embryos may provide hints for identifying candidate pNBs in the abdomen [12 NBs/hs in A1, four in A2 and three in A3 to A7 according to Truman and Bate (Truman and Bate, 1988)], and NBs that undergo PCD in the thorax at the end of embryogenesis [seven NBs/hs in T1 to T3 (Truman and Bate, 1988)]. In the abdomen, NB1-1a and NB6-2 are obvious candidates for pNBs, as they remained consistently unchanged in H99 embryos (Table 1). Two other NBs, NB1-2 and NB3-2, are also potential abdominal pNBs as they mostly did not differ from their wt counterparts, and only occasionally contained one additional cell. On the other hand, clones which showed more than twice the cell number in H99 NB2-1, NB5-4a and NB7-3, see Table 1) than in wt, strongly suggest that these NBs normally undergo PCD in the abdomen (but perform additional divisions in H99), because, even if one daughter cell of each GMC undergoes PCD, they still cannot account for all cells found in H99 clones.

Regarding thoracic NBs, we can only speculate on account of low sample numbers. NBs which seem to become pNBs in the thorax, as they showed no difference between wt and H99 clones, are NB3-2 (n=2 clones in H99), NB4-3 (n=2) and NB4-4 (n=3) (Table 1). Potential candidates for NBs which do not become pNBs, but undergo PCD in the thorax, are expected to consistently have a...
significant increase in cell number in H99. These are NB5-1 (n=2 clones in H99) and NB5-5 (n=5) (Table 1). In addition, lineages for which we obtained only one clone in H99 but which also showed many more cells in the thorax than normal are NB2-2t, NB5-4t and NB7-3 (Table 1, and see Fig. S2 in the supplementary material).

Established models for studying the mechanisms of developmental PCD in the CNS

In order to investigate the developmental signals and mechanisms involved in the regulation of PCD in the embryonic CNS, we identified some of the apoptotic cells which will be used as single-cell PCD models. These are the DHb9-positive RP neuron from NB3-1, Lbe-positive neurons from NB5-3, the Eg-positive GW neuron from NB7-3 and the Eve-positive U neurons from NB7-1. As not much is known about the dying RP motoneuron or the Lbe-positive neurons, our first goal is to characterize each of these cells more closely, based on the combination of expressed molecular markers.

Some of the dying NB7-3 cells are already known to be undifferentiated daughter cells of the second and third GMC, which undergo PCD shortly after birth. Notch has been identified as the signal initiating PCD. The surviving daughters receive the asymmetrically distributed protein Numb, which counteracts the PCD-inducing Notch signal (Lundell et al., 2003). The same had been shown in a sensory organ lineage of the embryonic peripheral nervous system, where cells produced in two subsequent divisions undergo Notch-dependent PCD (Orgogozo et al., 2002). Both the PCD in the NB7-3 lineage and in the sensory organ lineage require the Hid, rpr and grim genes (Novotny et al., 2002; Lundell et al., 2003; Karcavich and Doe, 2005; Orgogozo et al., 2002). It will be interesting to see whether the Notch-Numb interaction also plays a role in the segment-specific PCD of the differentiated GW motoneuron, or if another signal is used for the removal of this, and possibly other, differentiated cells.

The U motoneurons also show a segment-specific cell death pattern (they apoptose in A6 to A8), thus somewhat resembling the MP1 and dMP2 neurons (Miguel-Aliaga and Thor, 2004). However, in contrast to MP1 and dMP2, the U neurons survive in the anterior segments and undergo PCD in the posterior ones. Whether homeotic genes play any role in the survival or death of these cells remains to be investigated.

In summary, we present here descriptions of PCD in the developing CNS of the wt Drosophila embryo, and of the CNS of PCD-deficient embryos. We find the pattern of Caspase-dependent PCD to be partly very orderly, suggesting tight spatio-temporal control of cell death, and partly random, which suggests a certain amount of plasticity already in the embryo. The CNS of PCD-deficient embryos is nevertheless well organized, despite the presence of too many cells. We find these superfluous cells to come from both a block in PCD and from additional divisions that surviving NBs go through. We were able to link the occurrence of cell death to identified NB lineages by clonal analysis in PCD-deficient embryos, to uncover segment-specific differences, and to establish single-cell PCD models that will be used in further studies to investigate mechanisms responsible for controlling PCD in the embryonic CNS.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/02707/DC1

References


Miguel-Aliaga, I. and Thor, S. (2004). Segment-specific prevention of pioneer neurons from both a block in PCD and from additional divisions that surviving NBs go through. We were able to link the occurrence of cell death to identified NB lineages by clonal analysis in PCD-deficient embryos, to uncover segment-specific differences, and to establish single-cell PCD models that will be used in further studies to investigate mechanisms responsible for controlling PCD in the embryonic CNS.


