Macrophage-mediated corpse engulfment is required for normal Drosophila CNS morphogenesis

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SUMMARY

Cell death plays an essential role in development, and the removal of cell corpses presents an important challenge for the developing organism. Macrophages are largely responsible for the clearance of cell corpses in Drosophila melanogaster and mammalian systems. We have examined the developmental requirement for macrophages in Drosophila and find that macrophage function is essential for central nervous system (CNS) morphogenesis. We generate and analyze mutations in the Pvr locus, which encodes a receptor tyrosine kinase of the PDGF/VEGF family that is required for hemocyte migration. We find that loss of Pvr function causes the mispositioning of glia within the CNS and the disruption of the CNS axon scaffold. We further find that inhibition of hemocyte development or of Croquemort, a receptor required for macrophage-mediated corpse engulfment, causes similar CNS defects. These data indicate that macrophage-mediated clearance of cell corpses is required for proper morphogenesis of the Drosophila CNS.

Key words: Pvr, Engulfment, Hemocyte, Cell death, Apoptosis, Drosophila

INTRODUCTION

Programmed cell death plays an important role in sculpting tissues and neuronal circuitry during development (Jacobson et al., 1997). In Drosophila melanogaster, large numbers of cells die during development, and these deaths serve to shape tissue, to delete unnecessary structures, to control cell numbers and to eliminate damaged or developmentally defective cells (Abrams, 2002; Baehrecke, 2002; Bangs and White, 2000). For example, activation of cell death is essential for sculpting the embryonic head (Lohmann et al., 2002), eliminating cells at metamorphosis (Jiang et al., 1997), limiting the divisions of post-embryonic neuroblasts (Bello et al., 2003) and specifying the precise number of neurons in each cartridge within the lamina ganglion of the visual system (Huang et al., 1998). Such widespread occurrence of cell death creates many cell corpses, and the engulfment and removal of cell corpses is a prominent feature of animal development (Fadok and Chimini, 2001).

Macrophages are responsible for the majority of cell corpse removal during mammalian development (Hopkinson-Woolley et al., 1994; Hume et al., 1983; Morris et al., 1991). In Drosophila, cell corpse removal also relies on specialized phagocytic cells that resemble mammalian macrophages in a number of cellular and molecular properties (Franc, 2002). Drosophila macrophages are derived from hematopoietic precursor cells known as hemocytes, which differentiate into macrophages displaying phagocytic and scavenger properties in response to cell corpse exposure (Tepass et al., 1994). Work on mammalian macrophages and other phagocytic cells has identified several classes of receptors implicated in corpse recognition, including lectins, integrins, the MER tyrosine kinase, the phosphatidylserine receptor (PSR) and scavenger receptors such as CD36 (Fadok and Chimini, 2001). Drosophila macrophages express a CD36-related receptor, Croquemort (Franc et al., 1996), and croquemort (crq) function is required for macrophages to take up dead cells efficiently in the developing embryo (Franc et al., 1999).

As macrophages are responsible for much of the dead cell engulfment in developing animals, an important role for macrophages in tissue morphogenesis during development has been suggested (Morris et al., 1991). However, direct evidence of a required role for macrophage-mediated cell corpse engulfment in development is limited. In the feet of PU.1 mutant mice that lack macrophages, for example, other cell types take over the engulfing role and permit morphogenesis to proceed, albeit at a slower pace (Wood et al., 2000). By contrast, in the developing mouse retina macrophages are essential for cell death-mediated morphogenesis (Lang et al., 1994; Lang and Bishop, 1993). In this case, the primary defect is not caused by a failure of engulfment. Rather, macrophages are required to initiate the cell deaths that normally eliminate the hyaloid vessels and the pupillary membrane during the development of the mouse eye.

In the CNS of Drosophila melanogaster embryos, programmed cell death eliminates many neurons and glia (Jacobs, 2000; Sonnenfeld and Jacobs, 1995b). In the case of the midline glia, approximately ten midline glial cells are generated in each segment by stage 13 of embryonic...
development. As development proceeds, most of these glia are eliminated by programmed cell death, leaving two to three midline glia per segment by stage 17 (Klambt et al., 1991; Sonnenfeld and Jacobs, 1995a; Zhou et al., 1995). Recent work indicates that midline glial cell survival is mediated through activation of MAP kinase signaling in the midline glia via the reception of the EGFR ligand Spitz, which is provided by the developing neurons (Bergmann et al., 2002). In addition to midline glia, a subset of developing neurons and longitudinal glia (which flank the midline) are also removed through cell death (Hidalgo et al., 2001; Sonnenfeld and Jacobs, 1995b).

Electron microscopic studies by Sonnenfeld and Jacobs demonstrated that the majority of cell corpses are expelled from the CNS and engulfed by macrophages (Sonnenfeld and Jacobs, 1995b). Cell corpses can also be detected in glial cells both within and at the surface of the CNS, indicating that glial cells also contribute to removal of dead cells. These authors also examined macrophage-less embryos derived from Bic-D mothers and found an increase in the number of unengulfed cells within the CNS as well as increased numbers in subperineurial glial cells at the periphery of the CNS. However, embryos derived from Bic-D mutant mothers have widespread patterning defects (duplication of posterior structures at the midline) and therefore have defects in the CNS patterning similar to those of Pvr mutants. Taken together, these data suggest that macrophage-mediated engulfment is necessary for proper Drosophila CNS development.

MATERIALS AND METHODS

Genetics and molecular biology

Fly stocks and plasmids for creating the Pvr disruption allele were obtained from K. Golic. To create the targeting construct, bases 125741-130091 from AE903620 were excised as a SacII/BglII fragment and cloned into pTV2. An I-SceI recognition site was inserted at the BstEII site at base 128126. Targeting was done as described (Rong and Golic, 2000). EMS alleles were sequenced by the MGH DNA Sequencing Core Facility.

Genomic DNA was prepared for long-range PCR by crushing single flies (Canton-S, Pvr knock-out donor, or heterozygous Pvr knock-out) in 50 μl squishing buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 μg/ml Proteinase K). Crushed flies were incubated at 37°C for 30 minutes and Protease K was inactivated at 95°C for 2 minutes. Long-range PCR was performed using the Expand Long Template PCR System (Roche). Primers used to confirm homologous recombination were 5'-AATCGTACGGTGGGAATA-GTTGGG-3' (Primer A), 5'-AGAAGCGGAGGAGTTTTGGCAC-GAC-3' (Primer B), 5'-TTTGTCCAGACCTTGGAGACGAC-3' (Primer C) and 5'-TGTAAGAAGTCCCATCAACACCGGGC-3' (Primer D). Control PCR was performed with primers against Pvr genomic DNA, 5'-CAGTGCAACGCTAAGTGAGCC-3' and 5'-TC-TTCACGGAATAGTGGTCG-3'.

The following fly stocks were kindly provided by those indicated: slit(1.0)-lacZ (I. Rebay), Sim-Gal4 (C. Goodman), Gcm-Gal4 (U. Tepass), UAS-PvrDN (F. Rorth), srpneo5 (U. Barnerjee) and Df(3LR)H99 (Bloomington Stock Center). Homozygous Pvr and srpneo5 mutant embryos were identified through the use of balancers marked with GFP (Bloomington Stock Center).

Southern blotting

Genomic DNA isolated from 25 Canton-S or Pvrko/Cyo flies was digested with PsvII. Digested DNAs were electrophoresed on an 0.8% agarose gel and transferred by downward capillary transfer to a Zeta-Probe GT membrane (BioRad). Membrane was subsequently treated following manufacturer’s protocol. The probe template was amplified by PCR using genomic DNA and primers 5'-ATCGCTCTGTAGGCTAGCCTCAAAG-3' and 5'-CTCTTTCGCTAACAATCAGCACATT-3', which span 776 bases of the Pvr locus. 32P-labelled probe was made from this template using the DECAprime II Kit (Ambion).

Immunohistochemistry and western blotting

All embryos were stained as described (Pate! 1994) using: rat polyclonal antiserum against Repo (Campbell et al., 1994) (1:500); mouse monoclonal antibody against lacZ (1:100; Promega); mouse polyclonal antiserum against Peroxidasin (Nelson et al., 1994) (1:500) and rabbit polyclonal antiserum against Croquemort (Franc et al., 1999) (1:500). The mouse monoclonal antibodies BP102 and 1D4, developed by C. Goodman, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies labeled with HRP, FITC or Cy3 were obtained from Jackson Laboratories. Polyclonal antiserum against Pvr was produced in rats (Covance) against a 30 kDa peptide containing the 275 C-terminal amino acids of Pvr fused to a 6XHis tag. Anti-Pvr antiserum was used at 1:300 on embryos. Fluorescent images were obtained using a Nikon PCM2000 confocal microscope.

For each lane of western blots, 10 embryos of each genotype were homogenized in phosphate-buffered saline (PBS) (130 mM NaCl, 175 mM Na2HPO4, 60 mM NaH2PO4). Lysates were run on a 9% polyacrylamide gel and transferred to Hybond-P membrane (Amersham Pharmacia). Membranes were blocked in 5% nonfat milk and probed with anti-Pvr antiserum diluted 1:2000 and HRP-conjugated goat anti-rat antibody diluted at 1:5000. Blots were stripped and reprobed with rat monoclonal antibody against Elav to confirm that each Pvr mutant lane contained similar or greater amounts of total protein than wild-type control.

RNAi

Inhibition of Croq by RNAi was performed as described (Kennerdell and Carthew, 1998). Bases 681-1203 and 1209-1737 of Croq cDNA RE02070 (ResGen; GenBank Accession Number AY070904) were PCR-amplified using primer 5'-GAATATACGACTCTATATA-
GGGAGAGGGACTGATGCTATGAAAGCTG-3' with 5'-GAATTAAATACGACCTACAATGAGGAGAAGCCATCTGTAAGTCACGGAGACTC-3' and 5'-GAATTAAATACGACCTACAATGAGGAGAAGCCATCTGTAAGTCACGGAGACTC-3' and 5'-TTACACGGGCACGTACG-3' with 5'-GAATTAAATACGACCTACAATGAGGAGAAGCCATCTGTAAGTCACGGAGACTC-3'. The 5'-end of each oligonucleotide contains a T7 RNA polymerase promoter sequence. dsRNAs were synthesized with T7 RNA polymerase from the MEGAscript High Yield Transcription Kit (Ambion). dsRNA was injected into Canton-S embryos at 1 µg/µl. Both Crq dsRNAs gave similar results. Control embryos injected with dsRNA from the Gal4

RESULTS

Targeted disruption of the Pvr locus

To initiate our studies of Pvr function, the Pvr locus was disrupted using a recently described procedure for gene disruption by homologous recombination (Fig. 1A) (Rong and Golic, 2000; Rong and Golic, 2001). Ten independent homologous recombination events were recovered from the progeny of 3000 females, and the products of the targeting events were examined by a combination of long-range PCR and Southern blotting. Nine out of ten targeted insertions, including PvrKO2, yield the products expected for a successful targeting event in both PCR and Southern blot analysis (Fig. 1B,C). These alleles all failed to complement the lethality of Df(2L)TE128x11, a chromosomal deficiency that removes Pvr, completing embryogenesis but failing to hatch. One targeted insertion allele, PvrKO8, appeared to have undergone a complex combination of DNA insertion and duplication and was not analyzed further.

In addition to creating targeted insertions at the Pvr locus, we identified multiple Pvr alleles though a genetic non-complementation screen. Nine-thousand eight-hundred and ten lines of EMS-mutagenized flies were screened for failure to complement the lethality of Df(2L)TE128x11. One-hundred and eighty-five lethal or semi-lethal lines were recovered, of which 20 failed to complement PvrKO lethality. Among these putative Pvr alleles, we have identified four that carry missense mutations in the extracellular domain, four that create stop codons in the extracellular domain and three that carry missense mutations in the kinase domain (Fig. 2A). The mutations in the kinase domain disrupt residues highly conserved among protein kinases (Hanks et al., 1988; Johnson et al., 1996). In Pvr729 an alanine residue in the catalytic loop, highly conserved among tyrosine kinases, is changed to a

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Pvr is required for proper CNS axon scaffold formation and glial positioning

We next examined CNS patterning in Pvr mutants. CNS axons in the Drosophila embryo establish a precise pattern reiterated in each segment (Fig. 3A). CNS axons establish two longitudinal tracts that run the length of the embryo on either side of the midline, with a subset of these axons crossing the midline of the embryo, forming two commissural axon bundles per segment. CNS axons are guided in part by signals from glia precisely positioned at the midline and along the longitudinal tracts (Hidalgo and Booth, 2000a; Kidd et al., 1999). Although CNS axon architecture was grossly normal in Pvr mutants, the precise ladder-like axon scaffold seen in wild-type embryos was disrupted (Fig. 3B). The scaffold in each segment had a rounded appearance, owing to changes in the separation between the anterior and posterior commissures and the longitudinal tracts.

As a metric of CNS shape change in Pvr mutants, we calculated the ratio of the distance between the longitudinal tracts in each segment of late stage 16 embryos to the distance between anterior and posterior commissures. As shown in Table 1, this ratio was significantly smaller in Pvr mutants than in wild-type or Pvr heterozygote controls (unpaired t-test, P<0.01).

As glial cells within the CNS are required for proper axon tract formation, we examined the positioning of glial cells in Pvr mutants. Many CNS glial cells express the homeobox protein Repo and require Repo for their proper development (Campbell et al., 1994; Halter et al., 1995). Repo-expressing glia form a patterned array along the longitudinal axon tracts and are largely excluded from the midline, except for a thin line of glia that enter the midline in each embryonic segment (Campbell et al., 1994; Jacobs and Goodman, 1989) (Fig. 3C, arrowhead). In Pvr mutants, however, large numbers of Repo-expressing glia accumulated in the midline (Fig. 3D, arrowheads). Thus, Pvr mutants have defects in both CNS axon tract morphology and glial positioning.

As Pvr mutants showed disruptions in CNS axon tract shape and glial cell positioning near the CNS midline, the pathfinding
of CNS axons near the midline in Pvr mutants was examined in greater detail. The monoclonal antibody 1D4 (mAb 1D4) recognizes the Fasciclin 2 protein (Van Vactor et al., 1993) and labels a subset of longitudinal bundles that grow adjacent to the CNS midline. Mab1D4 is a commonly used tool for assessing axon fasciculation patterns and detecting inappropriate axon crossing of the CNS midline (Hidalgo and Brand, 1997; Kidd et al., 1998; Lin et al., 1994). Despite the changes in CNS axon scaffold shape and longitudinal glial distribution observed in Pvr mutants, no inappropriate axon crossing of the midline was detected (Fig. 3E,F). In addition, in wild-type animals three major tracts of Fasciclin 2-positive axons are observed near the dorsal surface of the CNS on either side of the midline (Fig. 3E). Three major tracts of Fasciclin 2-positive axons were also observed in Pvr mutants (Fig. 3F).

The Pvr-expressing cells at the CNS midline were midline glia (a population distinct from the Repo-positive glia mentioned above) and were intimately associated with the CNS midline (Hidalgo and Booth, 2000b), acting in midline glia, high-level expression of a dominant-negative form of Pvr (Duchek et al., 1999) disrupted axon fasciculation between the commissures (Fig. 4E,F). Pvr expression could not be detected in PvrKO2, Pvr5363 or Pvr5742 embryos, confirming the specificity of the antisera (Fig. 4G).

**Drosophila** midline glia play important roles in separating and wrapping CNS axon commissures (Jacobs, 2000). Although midline glia expressed Pvr and Pvr mutants exhibited defects in commissure morphology, we could detect no role for Pvr in the midline cells. We first examined the number of midline glia present in Pvr mutant embryos using the midline glial marker slt(1.0)-lacZ (Nambu et al., 1991). Wild-type stage 17 embryos contained 2.8±0.2 glia per segment (n=18±s.e.m.), while PvrKO2 embryos contained 2.7±0.2 glia per segment (n=25). Thus, there was no detectable alteration in the number of midline glia. Midline glial cell development was further examined by staining for Wrapping, an immunoglobulin superfamily protein specifically expressed in midline glia and required for midline glial survival (Noordermeer et al., 1998). Wrapping was appropriately expressed by midline glia in Pvr mutants (Fig. 5A,B). To test directly whether Pvr acted in midline glia, high-level expression of a dominant-negative form of Pvr (Duchek et al., 2001) was driven in all midline cells using the Sim:Gal4 driver or specifically in midline glia using the Slit:Gal4 driver. However, in neither case was a detectable CNS axon or Repo-positive glia phenotype generated (Fig. 5C,E; H.C.S., C.J.K. and P.A.G., unpublished).

We next examined whether the CNS phenotype was related to Pvr expression in hemocytes. In the Pvr alleles recovered in our studies, hemocytes largely failed to migrate away from

*Table 1. Quantitative representation of CNS commissure morphology of late stage 16/early stage 17 embryos*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ratio of distance between longitudinal axon bundles and commissures*</th>
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<tr>
<td>+/-</td>
<td>2.5±0.09 (n=40)</td>
</tr>
<tr>
<td>PvrKO2/+</td>
<td>2.6±0.09 (n=40)</td>
</tr>
<tr>
<td>PvrKO2/0</td>
<td>1.6±0.06 (n=40)</td>
</tr>
<tr>
<td>Pvr5363</td>
<td>1.7±0.07 (n=32)</td>
</tr>
<tr>
<td>syp5045</td>
<td>1.6±0.08 (n=40)</td>
</tr>
<tr>
<td>crq RNAi</td>
<td>1.8±0.07 (n=32)</td>
</tr>
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*In each embryonic segment, the distance between the longitudinal axon bundles and the distance between the commissural axon bundles was measured, and the ratio of the two quantities was calculated. A ratio was used so that variations in overall embryo size between individuals would not contribute to the result. Error is given as standard error of the mean.† Significantly different from wild type, P<0.01 (unpaired t-test). n=number of segments quantitated.
their birthplace in the head (Fig. 5G,H), consistent with the recent observations of Cho et al. (Cho et al., 2002). To test whether Pvr function in hemocytes was important for CNS development, we drove expression of dominant-negative Pvr in the developing hemocytes. While no solely hemocyte-specific Gal4 driver is available, Gcm:Gal4 has been used previously to drive gene expression in embryonic hemocytes (Cho et al., 2002). We find that Gcm:Gal4 drives gene expression specifically in hemocytes beginning at stage 11 and later, beginning at stage 15, in other cells that do not detectably express Pvr (H.C.S., C.J.K. and P.A.G., unpublished). Embryos expressing dominant-negative Pvr under the control of Gcm:Gal4 had hemocyte migration defects resembling those of Pvr mutants, consistent with Pvr acting cell-autonomously to control hemocyte migration (Fig. 5I). Most importantly, embryos expressing dominant-negative Pvr under Gcm:Gal4 control also exhibited rounding of CNS axon commissures and mispositioning of Repo-positive glial cells similar to Pvr mutants (Fig. 5D,F). These data are consistent with Pvr acting in hemocytes to control CNS patterning and suggest that hemocyte function is required during CNS development.

Fig. 4. Pvr protein is present on midline glia and hemocytes. (A-D) Wild-type stage 16 embryo stained with anti-Pvr (red) and anti-Peroxidasin (Pxn) (green). Yellow indicates overlap in the merged images (C,D). Pvr protein can be detected at the midline (arrowheads), in hemocytes (arrows) and at the surface of the embryo. (D) Higher magnification image of embryo depicted in A-C. The expression of Pvr in hemocytes is confirmed by co-expression with Peroxidasin in C and D. (E) Stage 17 embryo double-labeled with anti-Pvr (black) and BP102 (brown). Pvr protein is expressed on midline glia that surround the CNS commissures (arrowhead). (F) Higher magnification image of wild-type stage 16 embryo stained with anti-Pvr. CNS axon tracts are visualized using DIC optics. Pvr protein is expressed on midline glia (arrowheads) that surround the anterior and posterior CNS commissures. ac, anterior commissure. pc, posterior commissure. lo, longitudinal axon tracts. (G) PvrKO2 mutant embryo stained with anti-Pvr. Anti-Pvr staining cannot be detected in mutant animals.

Fig. 5. Pvr acts in hemocytes and not midline glia for proper CNS patterning. (A,B) Stage 16 embryos stained with anti-wrapper antibody to visualize midline glia. Pvr5363 midline glia (B) are indistinguishable from wild type (A). (C,D) Stage 16 (C) Sim-Gal4;UAS-PvrDN and (D) Gcm-Gal4;UAS-PvrDN embryos stained with mAb BP102 to visualize CNS axons. Sim-Gal4;UAS-PvrDN embryos are indistinguishable from wild type, with commissures and longitudinal tracts forming a rectangular axon scaffold in each segment (arrow). Gcm-Gal4;UAS-PvrDN embryos resemble Pvr embryos, with the axon scaffold having a rounded appearance in each segment (arrow). (E,F) Stage 16 (E) Sim-Gal4;UAS-PvrDN and (F) Gcm-Gal4;UAS-PvrDN embryos stained with anti-Repo antibody to visualize glia. Sim-Gal4;UAS-PvrDN embryos have glia in normal positions, with only few glia located between commissures in each segment (arrowhead), while many segments of Gcm-Gal4;UAS-PvrDN embryos have clusters of glia near the midline (arrowheads). (G-I) Stage 16 (G) wild type (Canton-S), (H) PvrKO2 and (I) Gcm-Gal4;UAS-PvrDN embryos stained with anti-Croquemort to visualize hemocytes. In wild type, hemocytes are dispersed throughout the embryo, while in Pvr mutants and Gcm-Gal4;UAS-PvrDN embryos hemocytes are largely clustered near the dorsal and anterior regions of the embryo. Anterior is towards the left and dorsal is towards the top.
Macrophage engulfment is required for CNS patterning

To further examine the potential contribution of hemocytes to CNS development, we examined animals mutant for serpent (srp), which encodes a GATA-family transcription factor required for hemocyte development (Rehorn et al., 1996). srp<sup>neo45</sup> is a hemocyte-specific allele of serpent, and srp<sup>neo45</sup> animals lack all hemocytes (Lebestky et al., 2000; Rehorn et al., 1996). Examination of srp<sup>neo45</sup> embryos demonstrated that not only did srp<sup>neo45</sup> mutants lack macrophages, they also exhibited CNS axon scaffold defects similar to those in Pvr mutants, with characteristic rounding of commissures (Fig. 6C). Quantitative representation of CNS axon tract morphology in srp<sup>neo45</sup> mutants confirmed this observation (Table 1). The ratio of the distance between the longitudinal axon tracts and the distance between the commissural axon tracts in srp<sup>neo45</sup> mutants was significantly different from wild type (P<0.01), but not significantly different from Pvr homozygotes (P>0.2) (Table 1). srp<sup>neo45</sup> animals also showed longitudinal glia positioning defects similar to those seen in Pvr mutants (Fig. 6G). Thus, mutants that disrupt either hemocyte production or migration cause similar alterations in CNS morphogenesis.

One possible explanation for dependence of CNS morphogenesis on hemocytes is that hemocyte-derived macrophages are needed to engulf cell corpses generated during development. To test this possibility we examined animals in which macrophages appear to develop normally, but fail to engulf cell corpses. This was achieved using animals with reduced function of crq, which encodes a CD36-related receptor required for Drosophila macrophages to engulf dead cells (Franc et al., 1999). Previous genetic studies of crq function used the chromosomal deficiencies Df(2L)al and Df(2L)TE99(Z)XW88. These deficiencies remove a number of genes, including u-shaped, a transcriptional regulator that acts in the hemocyte lineage and is involved in germ-band contraction (Fossett et al., 2001; Franc et al., 1999). As the elimination of multiple genes affecting potentially related processes complicates the use of Df(2L)al and Df(2L)TE99(Z)XW88 in the analysis of CNS development, crq loss-of-function was examined using RNAi. Embryos injected with dsRNA corresponding to either of two non-overlapping regions within the Crq transcript had CNS axon scaffold defects similar to those in Pvr and srp mutants (Fig. 6D). In crq RNAi embryos the ratio of distance between longitudinals to distance between commissurals was significantly different from wild type (P<0.01), but not significantly different from Pvr or srp mutants (P>0.2) (Table 1). In addition, crq RNAi animals also showed defects in the positioning of Repo-positive glia similar to those seen in Pvr and srp mutants (Fig. 6H). These data further support the importance of hemocytes in CNS morphogenesis and specifically suggest that engulfment of dead cells by hemocyte-derived macrophages is essential for CNS development.

**DISCUSSION**

We have examined the role of macrophage function in Drosophila development using several genetic approaches. Through a combination of site-specific gene targeting and chemical mutagenesis we generated and characterized a series of mutations in Pvr, which encodes a receptor tyrosine kinase of the PDGF/VEGF receptor family. In addition to disrupting macrophage migration, a function of Pvr recently described by Cho et al. (Cho et al., 2002), we find that Pvr mutants have defects in glial positioning and axon scaffold formation in the CNS. Tissue-specific expression of dominant-negative Pvr suggests that the CNS defects result from the disruption in macrophage positioning. Consistent with this interpretation, similar defects in CNS patterning are observed in animals that completely lack macrophages (srp<sup>neo45</sup> mutants) and in animals in which expression of Croquemort (Crq), a receptor required for macrophage engulfment of dead cells, is inhibited. Taken together, these data suggest that macrophages play an important role in CNS development. Furthermore, as the effects on CNS morphogenesis of inhibiting croquemort function are essentially indistinguishable from eliminating macrophages altogether, our data suggest that corpse engulfment is a major aspect, and perhaps the most important aspect, of macrophage function in CNS development.

There are several possible explanations for the observed contribution of macrophages to CNS morphogenesis. As engulfment is capable of promoting cell death in some situations (Reddien et al., 2001), the inhibition of macrophage function could potentially change patterns of cell death. However, previous work found that substantial cell death still occurs in Drosophila embryos in the absence of macrophages (Tepass et al., 1994). Similarly, we find no alteration in the number of midline glia in Pvr mutants, suggesting that the...
death of midline glia proceeds normally. Acidine Orange staining of developing embryos likewise shows no detectable alteration in the pattern of dead cell generation (H.C.S., C.J.K. and P.A.G., unpublished). Thus, although subtle changes in pattern of cell death would escape detection by these methods, there is no large-scale alteration in cell death in Drosophila in the absence of macrophages. Interestingly, when cell death is blocked in homozygous Df(3L)H99 animals, which lack the cell death promoting genes hid, grim and reaper, the CNS axon scaffolds are wider than normal (Zhou et al., 1995) (H.C.S., C.J.K. and P.A.G., unpublished). The phenotypic contrast between the absence of cell death and the absence of macrophages suggests that macrophages are not simply required to remove material from the developing midline.

An alternative explanation for the need for macrophage-mediated engulfment is that the accumulation of cell corpses within the CNS disrupts axon and glial positioning. Cell corpses could exert a toxic effect or could disrupt the function of particular cell populations by abnormally accumulating within these cells. Such possibilities are consistent with Sonnenfeld and Jacobs’ observation that in embryos from Bic-D mutant mothers (which, in addition to severe embryonic patterning defects, lack macrophages) cell corpses accumulate at the CNS periphery and in CNS glial cells (Sonnenfeld and Jacobs, 1995b). In the case of glial cells, it is interesting to note that the CNS defects of Pvr, serpentneo45 and crq RNAi animals resemble those of repo mutant animals in which glial positioning and survival is disrupted (Campbell et al., 1994; Halter et al., 1995). Thus, a disruption in glial positioning could lead to the disruption of the CNS axon scaffold observed in the absence of macrophage-mediated engulfment.

Another possible requirement for the engulfment of dead cells by macrophages could be that engulfment stimulates the release by macrophages of factors required for proper CNS morphogenesis. Drosophila macrophages produce extracellular matrix components such as collagen IV, laminin, papilin, glutactin and macrophage-derived proteoglycan-1 (MDP-1) (Fessler et al., 1994; Gullberg et al., 1994; Hortsch et al., 1998), and the presence of cell corpses is known to enhance production of at least one of these, MDP-1 (Hortsch et al., 1998). That Pvr, serpentneo45 and crq RNAi animals all show a mild elongation of the nerve cord (H.C.S., C.J.K. and P.A.G., unpublished) could reflect defects in extracellular matrix production.

Cell death is a major component of many morphogenetic processes during development in vertebrates and invertebrates. However, an essential role for macrophages in these morphogenetic events has been established only in the mouse retina, where macrophages appear to act by triggering cell death (Lang et al., 1994; Lang and Bishop, 1993). Unlike the mouse retina, cell death in the Drosophila CNS does not require macrophages (Tepass et al., 1994). Our data support a different role for macrophages in Drosophila CNS morphogenesis: mediating clearance of dead cells. These observations indicate that both cell death and the interaction of macrophages with cell corpses are required for proper Drosophila CNS development.

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