Development 130, 1505-1513

doi:10.1242/dev.00377

The *dead ringer/retained* transcriptional regulatory gene is required for positioning of the longitudinal glia in the *Drosophila* embryonic CNS

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Accepted 30 December 2002

SUMMARY

The *Drosophila dead ringer* (*dri*, also known as *retained*, *retn*) gene encodes a nuclear protein with a conserved DNA-binding domain termed the ARID (AT-rich interaction domain). We show here that *dri* is expressed in a subset of longitudinal glia in the *Drosophila* embryonic central nervous system and that *dri* forms part of the transcriptional regulatory cascade required for normal development of these cells. Analysis of mutant embryos revealed a role for *dri* in formation of the normal embryonic CNS. Longitudinal glia arise normally in *dri* mutant embryos, but they fail to migrate to their final destinations. Disruption of the spatial organization of the *dri*-expressing longitudinal glia accounts for the mild defects in axon fasciculation observed in the mutant embryos. Consistent with the late phenotypes observed, expression of the *glial cells missing* (*gcm*) and *reversed polarity* (*repo*) genes was found to be normal in *dri* mutant embryos. However, from stage 15 of embryogenesis, expression of *locomotion defects* (*loco*) and *prospero* (*pros*) was found to be missing in a subset of LG. This suggests that *loco* and *pros* are targets of DRI transcriptional activation in some LG. We conclude that *dri* is an important regulator of the late development of longitudinal glia.

Key words: Gene regulation, Embryogenesis, Glia, CNS, *Drosophila*, ARID motif, *retained*, *dead ringer*

INTRODUCTON

In animals, the initial development of the central nervous system (CNS) occurs during embryogenesis when neurons send out their processes, axons, to establish a stereotypical arrangement of axon bundles. Glial cells constitute the second major component of the nervous systems of all multicellular organisms. The function of the nervous system as a whole depends on the correct specification and function of both neurons and glia. Glia are essential for controlling proliferation rates of vicinal neuroblasts (Ebens et al., 1993) and for the correct establishment and survival of the axon scaffold (Booth et al., 2000; Hidalgo and Booth, 2000; Hidalgo and Brand, 1997; Hidalgo et al., 1995; Xiong and Montell, 1995). Furthermore, glia ensheath axon bundles, providing electro-insulation and spatial separation from neighbouring haemocytes, thereby creating the blood-brain barrier (Granderath et al., 1999b).

The two characterized classes of glial cells studied so far in the embryonic CNS of *Drosophila* are the midline glia (proposed to correspond to the floor plate of vertebrates) and longitudinal glia (reminiscent of vertebrate oligodendrocytes). These cells are distinguished on the basis of their different positions and patterns of gene expression. Midline glia, which originate from the mesectoderm, depend on expression of the *single-minded* (*sim*) gene and activation of the EGF signalling pathway for their development (Hummel et al., 1999; Klambt et al., 1991; Stemerdink and Jacobs, 1997). The ultimate phenotype of embryos mutant for genes essential for midline development is the absence of axon commissures and collapse of the longitudinal axon connectives.

Longitudinal glia are a heterogenous population of cells, the specification of which depends on the activity of the gene *glial cells missing/glial cell deficient* (*gcm/glide*). *gcm* encodes a transcription factor which binds to a conserved eight nucleotide DNA-binding motif called the GCM motif (Akiyama et al., 1996; Bernardoni et al., 1998; Hosoya et al., 1995; Jones et al., 1995; Schreiber et al., 1997; Vincent et al., 1996). GCM is a master regulator of glial development, acting as a switch between neural and glial cell fates. GCM performs its function through activation of the gene *tramtrack* (*ttk*), which encodes a zinc-finger protein that acts as a repressor of neural cell fate (Badenhorst, 2001; Giesen et al., 1997; Van De Bor et al., 2000). Concomitantly with repression of the neural cell fate, GCM triggers expression of proteins that mediate glial cell differentiation. These include a GCM homolog GLIDE2, the ETS domain protein Pointed (PNT), the homeodomain protein Reversed polarity (REPO) and Locomotion defects (LOCO), a

regulator of G-protein signalling (Campbell et al., 1994; Granderath et al., 1999b; Halter et al., 1995; Kammerer and Giangrande, 2001; Klaes et al., 1994; Klambt, 1993; Klambt and Goodman, 1991; Xiong et al., 1994). Activation of the expression of another glial-specific homeodomain protein, Prospero (PROS), in a subset of LG (six out of ten LG per hemisegment), depends on the activity of the DROP/MSH/Lottchen protein (DR – FlyBase) (Buescher and Chia, 1997; Doe et al., 1991). In *gcm* and *glide2* mutants, all glial cells acquire a default neural cell fate (Akiyama et al., 1996; Hosoya et al., 1995; Jones et al., 1995; Kammerer and Giangrande, 2001). Surprisingly, when PNT, REPO, LOCO or PROS functions are abrogated, longitudinal glia are still formed, albeit in a spatially disorganized fashion, indicating a failure to undergo terminal differentiation (Buescher and Chia, 1997).

Longitudinal glial cells are involved in the formation of the longitudinal axon fascicles by aiding navigation of the pioneer axon growth cones and by directing the fasciculation and defasciculation of axons (Hidalgo and Booth, 2000). Moreover, longitudinal glia are essential for follower axon survival during *Drosophila* embryogenesis (Booth et al., 2000).

The loss of correctly specified glia in either *gcm, glide2* or *Drop/MSH/ltt* mutant embryos accounts for the severe defects in the longitudinal connectives observed in these embryos (Akiyama et al., 1996; Buescher and Chia, 1997; Hosoya et al., 1995; Jones et al., 1995; Kammerer and Giangrande, 2001). By contrast, mutations in any of the *pnt*, *repo*, *loco* and *pros* genes result in fasciculation defects that are much weaker than those observed in the *gcm* mutant embryos (Buescher and Chia, 1997; Granderath and Klambt, 1999a). Embryos lacking any of these gene products develop with minor defasciculation of the longitudinal connectives.

Despite some progress in understanding the specification of the longitudinal glia, many genes involved in this process remain unidentified and the role of some of the known genes is not fully elucidated (Egger et al., 2002). One candidate for a role in embryonic CNS development is the *Drosophila dead ringer/retained* gene (*dri/retn*, here referred to as *dri*) (*retn* – FlyBase), because of its expression in the embryonic CNS (Gregory et al., 1996). *dri* encodes a nuclear protein with a conserved DNA binding domain termed the ARID [AT-rich interaction domain (Gregory et al., 1996) (reviewed by Kortschak et al., 2000)]. Analysis of the early stages of *Drosophila* embryogenesis showed that DRI is involved in aspects of dorsal/ventral and anterior/posterior axis formation acting either as a repressor or an activator depending on a particular developmental context (Valentine et al., 1998; Hader et al., 2000; Shandala et al., 1999). Later in embryogenesis, *dri* is required for myogenesis and hindgut development (Shandala et al., 1999).

We describe a role for *dri* in the formation of a functional CNS during *Drosophila* embryogenesis. We show that *dri* is expressed in a subset of glial cells as part of the cascade of transcriptional regulation that occurs during glial cell differentiation. *dri* mutant embryos are shown to exhibit mild defasciculation defects, similar to those caused by LG differentiation defects in *pnt*, *repo*, *loco* and *pros* mutant embryos. Finally, the *dri* mutant longitudinal glia are shown to be defective in their ability to migrate along the longitudinal axonal tracts to their proper positions, providing a cellular basis for the observed axon phenotype.

MATERIALS AND METHODS

Fly stocks

Two protein null alleles, dri^1 and dri^2, described previously (Shandala et al., 1999), were used during the course of this work. Enhancer trap and reporter lines used were: AA142, which labels midline glia; X55, which labels midline neurons (Klambt et al., 1991); *gcm* enhancer trap lines rA87 (Jones et al., 1995; Vincent et al., 1996) and gcm^P (Hosoya et al., 1995); rc56, a *loco* enhancer trap line (Klaes et al., 1994); and P2333, a *Star* enhancer trap (Spradling et al., 1999). For targeted expression of *dri* in the embryonic CNS, the yeast Gal4-UAS system was used (Brand and Perrimon, 1993). A previously reported UAS-*dri* construct (Shandala et al., 1999) was placed under the control of several Gal4-expressing constructs: *Kr*Gal4 (which expresses GAL4 in T2-A4), *sim*Gal4 (which expresses GAL4 in the embryonic midline, provided by C. Klambt, University of Muenster, Germany) and *MZ*1580 (a P*GAL4* insertion in the *actin* locus, which directs GAL4 expression in the longitudinal glia and its progeny as well as in MP2 neurons and macrophages, provided by G. Technau, Institute of Genetics, University of Mainz, Germany). The gcm^{P1} amorphic allele was provided by B. W. Jones (Skirball Institute, NYU School of Medicine, NY). Unless specified, fly stocks were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN).

Antibodies and staining methods

A rat polyclonal antibody raised against a bacterially expressed pGEX-DRI fusion protein (Gregory et al., 1996) was used to detect the distribution of DRI in embryos. Polyclonal anti-β-gal was obtained from Rockland Immunochemicals (Gilbertsville, PA), anti-REPO was provided by A. Travers (Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK), anti-Eve was provided by J. Reinitz (The University at Stony Brook, NY), the monoclonal antibodies 1D4 anti-FasII and 1B7 were provided by C. Goodman (University of California, Berkeley, CA), and anti-GFP was provided by P. Silver (Dana Faber Cancer Inst, Boston, MA) (Seedorf et al., 1999). The monoclonal antibodies 9F8A9 anti-ELAV, 2B10 anti-CUT, MR1A anti-PROS, 22C10, BP 102 and 4D9 anti-EN were all obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, USA). For immunohistochemistry, anti-IgG secondary antibodies conjugated with AP, HRP, Cy5 and Lissamine-Rhodamine (Jackson ImmunoResearch Laboratories, PA), and Alexa488 (Molecular Probes, OR) were used. Biotinylated anti-IgG secondary antibodies and AP-, Lissamine-Rhodamine-conjugated streptavidin (Jackson ImmunoResearch Laboratories, PA) were used where signal enhancement was required. Immunohistochemical staining was carried out according to the method of Foe (Foe, 1989). Embryos were mounted in phosphate-buffered saline (PBS) containing 80% glycerol and viewed with epifluorescence using a Zeiss Axiophot or by confocal microscopy using a BioRad MRC1000 scanhead equipped with a krypton/argon laser confocal microscope.

RESULTS

dead ringer is expressed in longitudinal glial cells

Gregory et al. have reported expression of *dri* in the CNS of *Drosophila* embryos (Gregory et al., 1996). To better characterize the pattern of expression, we co-stained embryos with the axon marker, monoclonal antibody 22C10 and anti-DRI antibodies. The staining revealed that some of the *dri*-expressing cells lie close to the midline (Fig. 1A, arrow). To further examine the nature of these cells, we used enhancer trap lines with previously described patterns of β-galactosidase expression in mesectoderm-derived cells: X55 (P element

insertion at 56E, expresses in the VUM neurones, the MNB and its support cells, and the posterior pair of the midline glia, Fig. 1B), P 2333 [a *Star* enhancer trap insertion (Spradling et al., 1999) (Fig. 1C)] and AA142 (a P element insertion at 66D that expresses β-galactosidase in the midline glia, MGA, MGM and MGP; Fig. 1D). No correspondence was observed between these markers and *dri*-expressing cells. However, using the midline-derived cell markers as positional landmarks, we could infer that the *dri*-positive cells correspond to dorsally located glial cells.

To confirm this, we employed specific molecular markers: the enhancer trap lines *rA87* (*gcm*, Fig. 2A,B), *pnt*^{rm254} (Fig. 2C) and *rC56* (*loco*, Fig. 2D), and the antibodies anti-REPO (Fig. 2E), anti-PROS (Fig. 2F) and anti-CUT (Fig. 2G). Co-expression of DRI with these glial markers confirmed that, with the exception of one cell per hemisegment at the very lateral edge of the ventral nerve cord (VNC, notched arrowheads in Fig. 1A-D), the *dri*-positive cells are longitudinal glia.

Based on their position within the VNC, the dorsal glia can be subdivided into the longitudinal glial (LG), the A and B subperineural glia (A/B SPG), which are positioned at the medial edge of the longitudinal glia, and the medialmost cell body glia (MM-CBG), which are located in the very medial position within the cortex (Halter et al., 1995; Ito et al., 1995; Jacobs et al., 1989; Klambt and Goodman, 1991; Udolph et al., 1993) (summarized in Fig. 2H). All LG, A/B SPG and MM-CBG express *gcm*, *repo* and *cut* (Fig. 2A,B,E,G). *dri* appears to be expressed in eight out of ten of the LG (Fig. 2, short arrows). It is known that the LG comprise eight cells that are the progeny of lateral glioblasts, plus approx. two cells per hemineuromere that are of unknown origin. The latter intermingle with other longitudinal glial cells and cannot be distinguished by their position or any molecular marker from stage 13 of embryogenesis (Halter et al., 1995; Ito et al., 1995;

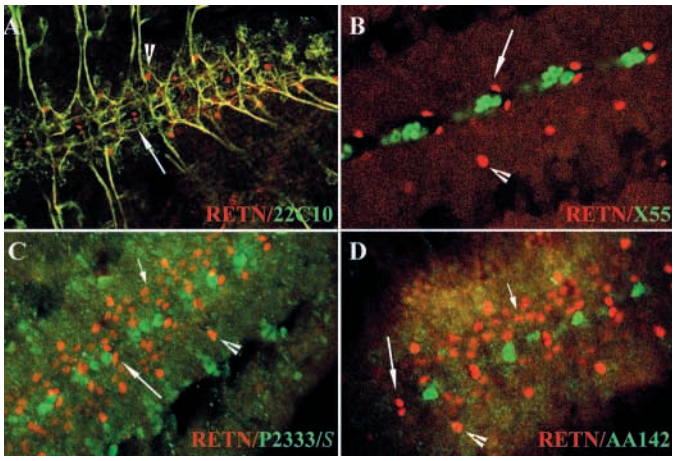

Fig. 1. *dri* is not expressed in midline-derived cells of the *Drosophila* embryonic CNS. Confocal micrographs of stage 15 whole-mount embryos carrying specific enhancer trap lines directing β-galactosidase expression in midline cells of the CNS. Embryos were immunochemically stained with polyclonal rabbit anti-β-gal (Alexa488-conjugated secondary antibody, green) and rat anti-DRI (Lissamine-Rhodamine-conjugated secondary antibody, red). Anterior is towards the left. Some *dri*-positive cells are located in the vicinity of the embryonic midline: (A) Posterior to the posterior commissures (arrow) as revealed by the mAb 22C10 axonal marker; (B) posterior to X55-positive cells (the VUM neurones, the MNB and its support cells and the posterior pair of the midline glia) (arrow); (C) posterior to *Star*-positive (P 2333) midline glia (long arrow); and (D) half way between neighbouring segmental clusters of AA142-positive midline glia (MGA, MGM and MGP) (long arrow). There is one *dri*-positive cell per hemineurome located at the very edge of the VNC (A-D, notched arrowhead), posterior to the intersegmental nerve root as revealed by the mAb 22C10 (A). Other *dri*-positive CNS cells are labelled with short arrows in C-D.

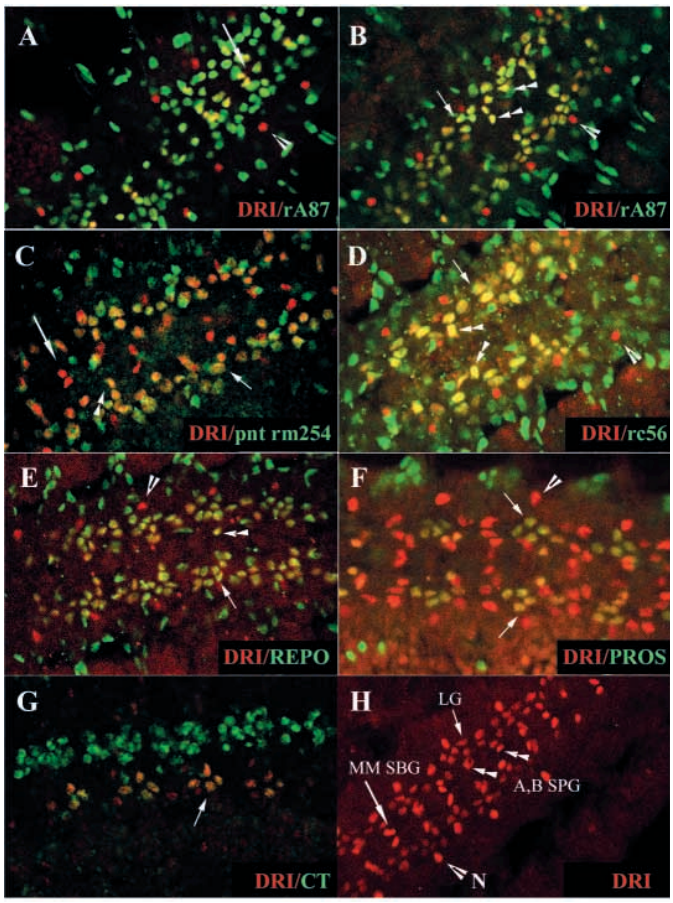

Fig. 2. *dri* is expressed in longitudinal glial cells of the *Drosophila* embryonic CNS. Confocal micrographs of stage 15 whole-mount embryos. Embryos are immunochemically stained with polyclonal rabbit anti-β-galactosidase (Alexa488-conjugated secondary antibody, green) and rat anti-DRI (Lissamine-Rhodamine-conjugated secondary antibody, red). Anterior is towards the left. (A,B) Focal planes showing that *dri* is expressed in the medialmost cell body glia (MM CBG, arrow in A), the subperineural glia (SPG, double arrowheads in B) and longitudinal glial (LG, short arrow in B) that express β-galactosidase in the rA87 enhancer trap line. (C) *dri* is expressed in the A and B SPG (double arrowhead) and LG (short arrow) that express β-galactosidase in the *pnt*^{rm254} enhancer trap line. (D) *dri* is expressed in the A and B SPG (double arrowheads) and LG (short arrow) that express β-galactosidase in the rC56 enhancer trap line. (E) *dri* is expressed in the A and B SPG (double arrowhead) and LG (short arrow) that express *repo*. (F) *dri* is expressed in all six *prospero*-expressing LG (short arrows, only five of these cells are in focus). (G) *ct* and *dri* are co-expressed in the LG (short arrow). (H) CNS stained for DRI alone showing the assignments of cells derived from the data shown in (A-F): LG (short arrow), A and B SPG (double arrowheads), MM CBG (long arrow) and N (neurone, notched arrowhead).

Jacobs et al., 1989; Klambt and Goodman, 1991; Udolph et al., 1993). Our data would be explained if *dri* is expressed in the eight progeny of the lateral glioblasts, but not in the two cells of unknown origin, but this is yet to be confirmed.

The A/B SPG and MM CBG express *dri* (Fig. 2). *pnt* and *loco* are expressed in the A/B SPG, but not the MM-CBG (Fig. 2C, data for *loco* are not shown). *pros* colocalizes with *dri* in only five LG per hemineuromere, but is absent from the A/B SPG and the MM-CBG (Fig. 2F).

In addition to the *dri*-positive glia, each hemineuromere has one lateral cell located posterior to the intersegmental nerve root (as revealed by the mAb 22C10 axon marker; Fig. 1A, arrowhead). The origin of these cells is unknown, but they do not express any glial specific marker (Fig. 2A-F) and they project axons along the longitudinal tracts (K.-L. Harris and P. Whitington, personal communication), confirming their identity as interneurons. The nature of these cells and the role of *dri* in their development is not considered further in this report.

dri is not involved in the neural/glial cell fate switch

Expression of *dri* in the embryonic CNS is first clearly detected at stage 11 in a single cell per hemineuromere, probably corresponding to the lateral glioblast (data not shown). DRI continues to be expressed while the daughter glial cells divide and migrate anteromedially (Fig. 3A).

The early expression of *dri* in a subset of longitudinal glia suggested that *dri* might be required for the initial neurone/glia fate decision. To test this, we first examined the distribution of *repo*-positive cells in *dri* mutant embryos from stage 11 up until stage14. The correct position and number of these *repo*-positive cells (Fig. 3B) suggested that initiation of gliogenesis is not affected in the absence of DRI.

We then tested whether ectopic expression of *dri* could induce glial fates in non-glial cells, as has been shown for *gcm* and *pnt* (Bernardoni et al., 1998; Klaes et al., 1994). However, *sim*-GAL4-induced expression of *dri* in midline cells was found not to increase the number of glial cells, as measured by the expression of *repo* (Fig. 3C, compare with 3D). Furthermore, when *dri* is driven by the MZ1580-GAL4 enhancer trap line that is specific for longitudinal glia and their progeny, as well as for MP2 neurones and macrophages, there were no ectopic *pros*-positive cells (data not shown). Ectopic expression of *dri* is therefore not sufficient to induce a glial cell fate.

Glial differentiation requires repression of the neuronal fate by the 69 kDa Tramtrack isoform (TTK69) (Badenhorst, 2001; Giesen et al., 1997). Overexpression of TTK69 in neuronal cells blocks neural differentiation. To test whether *dri* is capable of repressing the neural fate, we drove expression of UAS-*dri* in the T2-A4 segments using *Kr*-GAL4. In contrast to the effect of expressing TTK69, ectopic expression of DRI did not prevent normal neural differentiation in the CNS or PNS, as assayed by expression of the neuronal marker, ELAV (Fig. 3E, between arrowheads). The normal differentiation of neural cells also suggests that glial cell fate is not induced by DRI in the *Kr*-specific domain. Loss of TTK69 results in general derepression of the 22C10 neural specific antigen in somatic muscles (Giesen et al., 1997). In *dri* mutants, however, this antigen was observed in its normal pattern, i.e. in a single somatic muscle fibre (Fig. 3F). These results show that *dri* does not induce the glial fate, nor does it repress the neural cell fate.

Longitudinal axon tracts are defasciculated in *dri* mutant embryos

As the next step in exploring possible roles for *dri* in the LG, we investigated the organization of the longitudinal connectives. Axon projections along the VNC and the subsequent formation of longitudinal connectives depend on the proper function of longitudinal glia (Hidalgo and Booth, 2000; Hidalgo and Brand, 1997). During formation of the first longitudinal tract, LG navigate ipsilateral pioneer axons. Upon establishing contacts with axons, glia continue migrating along the longitudinal bundles, occupying choice points of axon fasciculation or defasciculation. The final fasciculation pattern can be visualized as three longitudinal Fasciclin 2 (Fas2)-positive (mAb1D4-staining) bundles on either side of the midline of stage 15-16 embryos (Grenningloh et al., 1991) (Fig. 4C).

In *dri* mutant embryos, the first processes projected by neurons were found to be normal (data

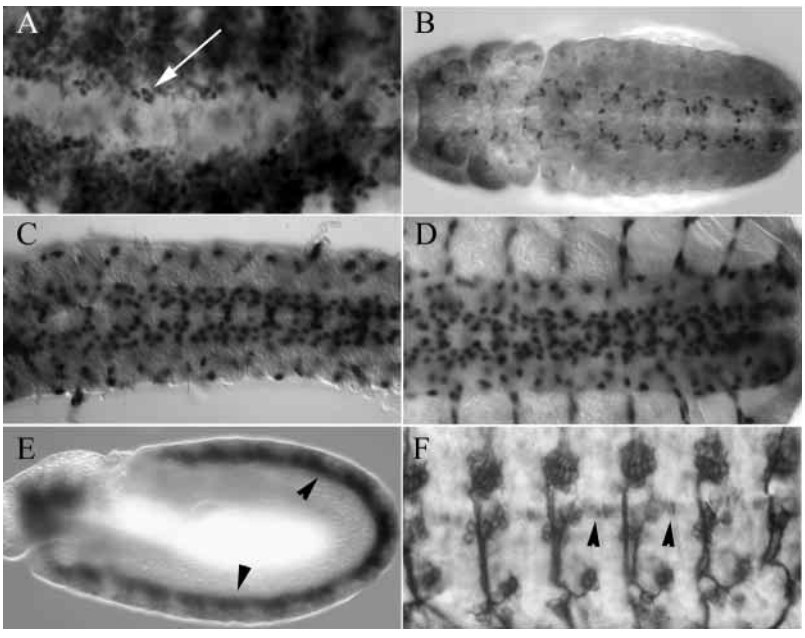

Fig. 3. DRI is not involved in initiation of the glial cell fate or repression of the neural cell fate decision in lateral glioblasts. (A) *dri* expression in longitudinal glia of a stage 12 embryo (arrow). (B) Whole-mount stage 12 dri^1/dri^2 embryo, derived from a maternal dri^1 homozygous germline clone, stained with anti-REPO antibody. Loss of maternal and zygotic DRI does not interfere with the early behaviour of the LG. (C) Wild-type stage 15 embryo stained with anti-REPO antibody. (D) Ectopic expression of *dri* in the mesectoderm using *sim-GAL4* does not induce longitudinal glial cell fate specification, as detected by anti-REPO staining (compare with C). (E) Ectopic expression of *dri* in parasegments T2-A4 using *Kr-GAL4* (between arrowheads) does not inhibit neural differentiation, as detected by anti-ELAV antibody. (F) 22C10 antigen is correctly localized in a single somatic muscle fibre in dri^1/dri^2 homozygotes (arrowheads).

not shown). This is expected, given that *dri* is not required for the specification of the longitudinal glia. Furthermore, at stages 11-14, no gross abnormalities were observed in the initial formation of the longitudinal connectives (Fig. 4B, compare with 4A). However, in stage 15-16 embryos, moderate but consistent fasciculation defects were observed in *dri* mutant embryos. Some axons were not bundled correctly within the fascicles, which appeared thinner and were occasionally found at the dorsal surface of the VNC (Fig. 4C-E). Axons were observed to occasionally join the wrong bundle or cross inappropriately between bundles, although they never crossed the midline. Moreover, the spacing between the first and second, and between the second and third parallel bundles was slightly increased. The severity of this phenotype was much less than that observed in *gcm* mutant embryos (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) (see Fig. 4G), but was similar to the level of disruption observed in *pnt* mutant embryos (Klaes et al., 1994; Scholz et al., 1993) (Fig. 4F).

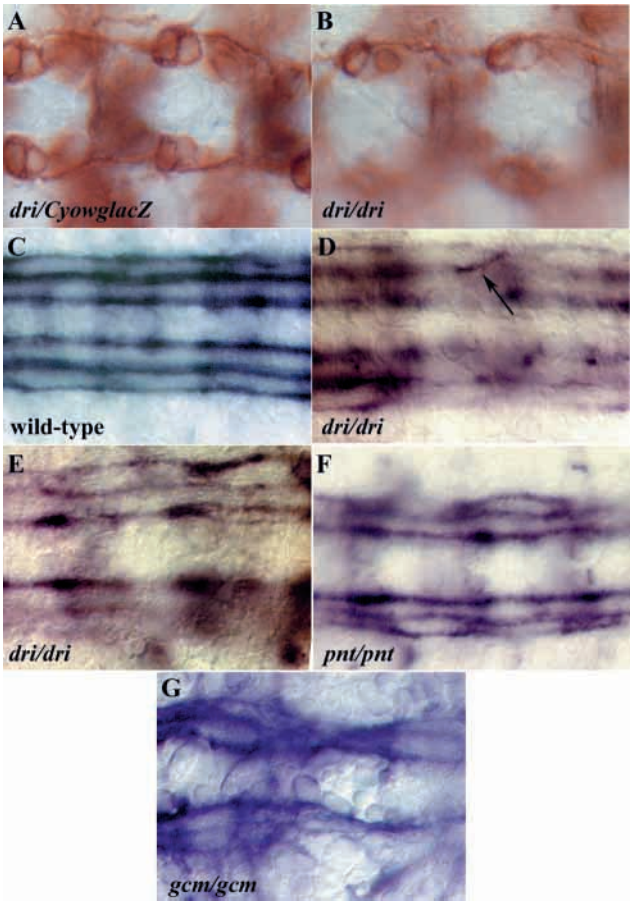

Fig. 4. Loss of DRI results in axon defasciculation in the *Drosophila* embryonic CNS. Dorsal view of dissected ventral nerve cord preparations. Anterior is towards the left. (A) The first axon bundle in control *dri* heterozygotes is revealed by anti-GFP staining of *ftz-GAL4*-driven UAS-*tauGFP*. (B) *ftz-GAL4*-driven UAS-*tauGFP* staining of a *dri* homozygous mutant embryo shows that the first axon bundle is correctly established. (C) Immunochemical staining with mAB 1D4, which recognizes the Fasciclin 2 protein, reveals the three axon bundles either side of the midline in a wild-type stage 16 embryo. (D) In dri^1/dri^2 mutant embryos, the first axon tract occupies the correct position relative to the midline, while the spacing between other tracts is slightly increased. Moreover, a moderate axon defasciculation is evident. In addition, axons occasionally cross between fascicles (arrow). (E) Another example of a dri^1/dri^2 mutant embryos, showing defasciculation of the three axon bundles. (F) pnt^{rm254} mutants exhibit a mild defasciculation of axons, similar to those observed in *dri* mutant embryos. (G) Strong pathfinding defects are observed in *gcmP1* mutant embryos.

This phenotype could arise as a consequence of neural or glial defects. With the aim of showing the glial-specificity of the *dri* phenotype, we attempted to rescue the axon defects by directing expression of UAS-*dri* in longitudinal glia using the MZ1580-GAL4 transgene (Hidalgo et al., 1995). Unfortunately, overexpression of *dri* interfered with normal glial development, causing misplacement of *pros*-positive cells and severe defasciculation of axons (data not shown). However, the possibility that the phenotype arose as a result of an indirect effect of DRI on neuronal development was ruled out by staining *dri* mutant embryos with anti-EVE, anti-EN, mAbBP102 and mAb22C10, and finding no defects in neural differentiation (data not shown).

Misplacement of longitudinal glial cells in *dri* mutant embryos

The fasciculation defects showed that the *dri* mutant longitudinal glia were defective in their axon guidance role. To understand the nature of this defect further, we examined the behaviour of these cells in *dri* mutant embryos. In wild-type stage16 embryos, the longitudinal glia form two flat sheets of cells either side of the midline underlying the dorsal surface of the axon tracts (Halter et al., 1995; Jacobs et al., 1989; Schmidt et al., 1997) (Fig. 5A,C,E). In *dri* mutant embryos, these cells were found to occupy a normal position relative to the midline, indicating that the LG had undergone their appropriate initial anterior-medial migration from their place of birth at the very lateral edge of the VNC (Fig. 5B,D). However, a pronounced defect in spatial organisation of the glia was observed. The mutant cells were ventrally shifted and not localized to the characteristic flat cell sheet on the dorsal side of the longitudinal connectives with only one or two out of ten glial cells per hemineuromere being in any one focal plane (Fig. 5F, compare with 5E).

Significantly, there is a high degree of similarity in the spatial disorganisation of the two rows of longitudinal glia in *dri* (Fig. 5F), *loco* and *pnt* mutant embryos (Granderath et al., 1999b). The *pnt* phenotype is associated with partial misregulation of *loco* expression, suggesting possible regulatory relationships between *pnt*, *loco* and *dri*, a possibility that is explored further below.

Longitudinal glia exhibit aberrant cell shapes in *dri* mutants

To characterize the cellular *dri* phenotype further, we used the *gcmP1* enhancer trap, which expresses β-galactosidase, and monoclonal antibody 1B7, which recognizes both the neuronal and non-neuronal form of the Neuroglian protein. Comparison between the wild-type (Fig. 6A-C) and *dri*-mutant (Fig. 6D-F) phenotypes of stage 14 CNS preparations revealed that some glia lose expression of the cell adhesion protein, Neuroglian (Fig. 6E,F, arrows). Significantly, Neuroglian-deficient glial cells show somewhat round shapes compared with the more elongated wild-type glia (Fig. 6, compare B with E). Misregulation of the cell adhesion protein Neuroglian might

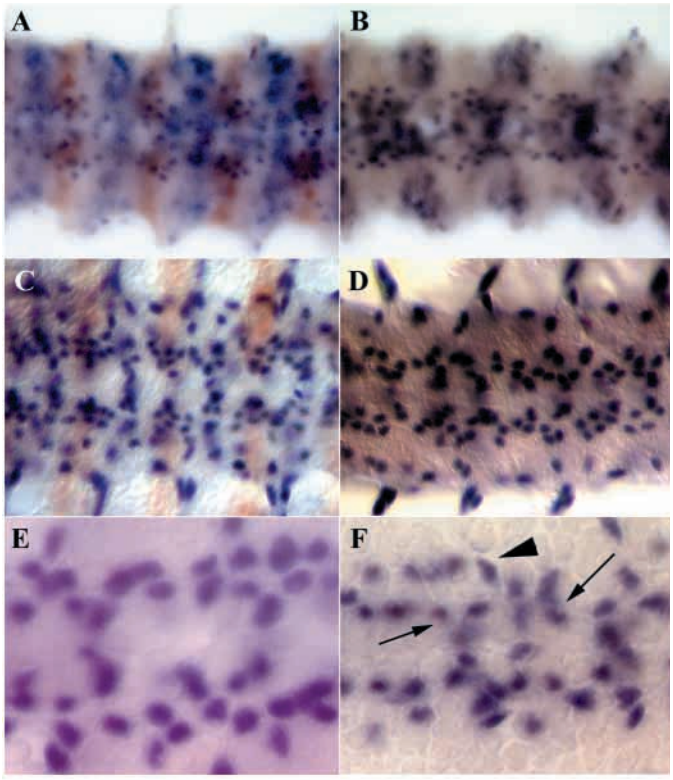

Fig. 5. The longitudinal glial cells expressing *repo* and *cut* are misplaced in *dri* mutants. Dorsal view of dissected ventral nerve cord preparations of *dri/CyO wgβ gal* (A,C,E) and dri^1/dri^2 homozygous (B,D,F) stage 16 embryos, immunochemically stained with anti-CT (A-B) or anti-REPO (C-F) antibodies and AP-conjugated (for REPO or CT in A-F) and HRP-conjugated (for β-gal in *CyO wgβ-gal,* A, C) secondary antibody. Anterior is towards the left. (A,C,E) *dri* heterozygotes exhibit the proper positions of two rows of glial cells either side of the midline. (B,D,F) Loss of DRI results in the misplacement of nuclei marked with CT (compare heterozygotes in A with *dri* homozygotes in B) or REPO (compare C with D). Higher magnification (E,F) more clearly shows that *repo*-positive nuclei of the longitudinal glial cells in *dri* homozygotes do not occupy a flat layer underlying the longitudinal connectives (F; arrowhead , nucleus in focus; arrows, mispositioned nuclei out of the focal plane) as in the wild type (E).

impede cell-cell contacts leading to cell shape changes and failure of glia to migrate normally along the axon tracts.

dri activity and the glial cell transcriptional regulatory cascade

As noted above, the similarity between the *dri* phenotypes and those of *repo*, *loco* and *pnt* suggested that gene regulatory relationships might exist between these genes. A considerable amount of information already exists about the nature of the transcriptional cascade required to establish longitudinal glial cells. The glial fate is induced by expression of *gcm*, while later expression of transcription factors encoded by *repo* and *pnt* direct glial differentiation (Akiyama et al., 1996; Bernardoni et al., 1998; Campbell et al., 1994; Hosoya et al., 1995; Jones et al., 1995; Klambt, 1993; Klambt and Goodman, 1991; Schreiber et al., 1997; Vincent et al., 1996; Xiong et al., 1994). We examined *dri* expression in embryos

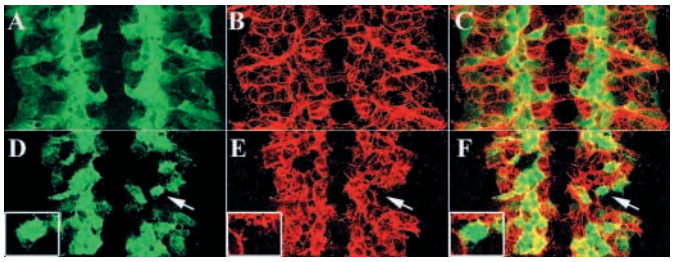

Fig. 6. Loss of DRI results in downregulation of Neuroglian in subsets of longitudinal glial cells. Whole-mount immunochemical staining of wild-type (A-C) and dri^1/dri^2 (D-F) embryos. Dorsal glial cells are labelled with *Pβ-gal* (driven by a *gcm* enhancer trap, green) and mAb 1B7 against NRG CAM (red). In all panels anterior is upwards. LG are slightly misplaced in some hemisegments. Some of those cells do not express Neuroglian and show a rounded cell shape (arrows in D,F indicate cells shown at higher magnification in inset), although the penetrance is low, the phenotype being detected on average in three hemisegments from T1 to A8 in an embryo.

mutant for genes required for glial formation and differentiation. As expected, *dri* expression in all of the dorsal glia (but not in the *dri*-expressing lateral neural cells) depends on *gcm* (Fig. 7B). Moreover, with the probable exception of the A/B SPG, the normal levels of *dri* glial expression requires *repo* (Fig. 7C), as *repo* mutant embryos show a significant reduction in the levels of *dri* expression and in the numbers of LG that contain DRI. However, *dri* expression in all dorsal glia does not depend on *pnt* (Fig. 7D). Analysis of the *dri* promoter region did not reveal any consensus binding sites for GCM (A/GCCCGCAT) or REPO (NNATTA, data not shown), suggesting that *dri* might be not a direct transcriptional target of these genes. The finding that *dri* expression in all glia is not affected in embryos mutant for *faint little ball* (*flb*), a null allele of the *Drosophila EGFR* gene (Fig. 7E,F), is in line with previous observations that only the midline glia require EGFR signalling.

In a complementary set of experiments, the expression of glial differentiation markers in a *dri* mutant background was examined. In *dri* loss-of-function mutants, *repo*, *pnt* and *cut* continue to be expressed in the appropriate glia (Fig. 5; data not shown). However, we observed a reduction in the number of *pros*- and *loco*-positive glial cells (Fig. 8). One *pros*-positive glial cell was found to be consistently missing in *dri* mutant embryos (depicted with arrows in Fig. 8A-D), while there was frequent, if irregular, reduction or loss of *pros* expression in three or four other LG (data not shown). Similarly, *loco* expression was reduced or lost in some LG, although this phenotype also exhibited variability in different segments (Fig. 8E,F). We scored the number of *loco*-positive cells in two neuromeres of abdominal segments in ten stage 15 embryos. The average number of dorsal glia per hemineuromere in $dri^1/CyOwglacZ$ heterozygotes was 9.8 (392 totally), not significantly different from wild-type numbers (Campbell et al., 1994; Halter et al., 1995; Klaes et al., 1994; Klambt, 1993; Klambt and Goodman, 1991; Xiong et al., 1994). By contrast, there was an average of 4.8 (193 scored) *loco*-positive cells per hemineuromere in dri^1 homozygotes, confirming the significance of the apparent loss of *loco*-expressing cells.

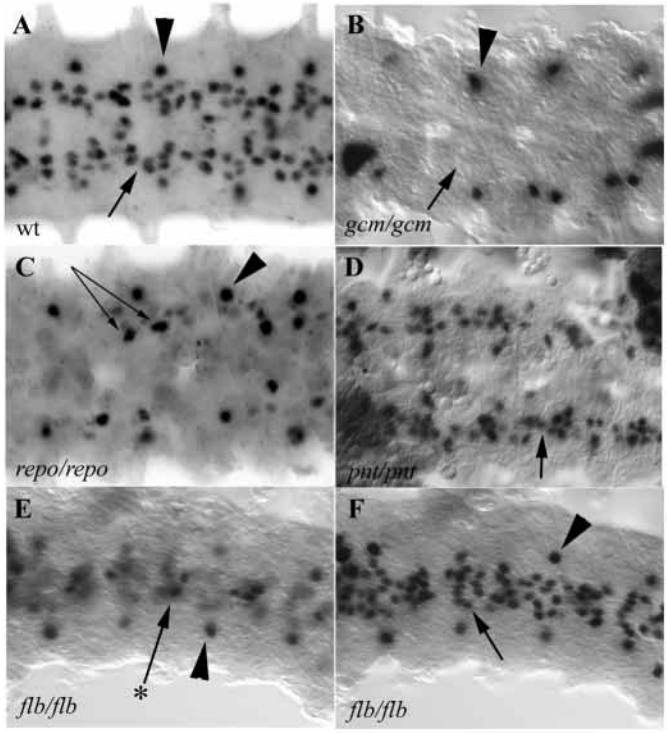

Fig. 7. *dri* expression in the longitudinal glial cells of the *Drosophila* embryonic CNS depends on GCM and REPO. Dorsal view of dissected embryonic ventral nerve cords immunochemically stained with anti-DRI and detected with AP-conjugated secondary antibody. Anterior is towards the left. (A) The distribution of *dri*-expressing cells in wild-type embryos. (B) *dri* expression is completely abolished in all glial (arrow) but not neural (arrowhead) cells in the *gcmP1* mutants. (C) *dri* expression is lost from most longitudinal glial cells but not from the neurone (arrowhead) nor, perhaps, from A/B SPG (thin arrows) in a $repo^{3}$ mutant embryo. (D) Expression of *dri* in the dorsal glia (arrow) seems to be unaffected in a stage 15 embryo mutant for $pnt^{\Delta 88}$. (E,F) Expression of *dri* in the dorsal glia is also unaffected in stage 15 embryos mutant for *flb*. (E) One focal plane, showing MM CBG, long arrow with asterisk. (F) A different focal plane showing the LG (short arrow) and the *dri*-positive neural cells (arrowhead).

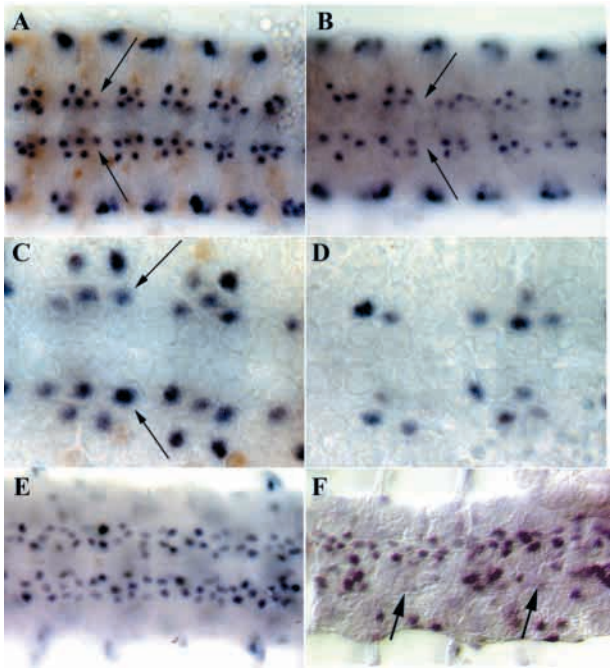

Fig. 8. The number of cells expressing *loco* and *pros* is reduced in *dri* mutants. Dorsal view of dissected ventral nerve cord preparations of *dri/CyO wgβ gal* (A,C,E) and dri^{1}/dri^{2} (B,D,F) stage 16 embryos, immunochemically stained with anti-PROS and anti-β-galactosidase (A-D), anti-β-galactosidase and anti-DRI antibodies (E,F) and detected with AP-conjugated (for PROS and rC56 β-gal; A-F) and, in some cases, HRP-conjugated (for β-gal: A,C; and DRI in E) secondary antibody. Anterior is towards the left. (A) In *dri/CyO* embryos there are five *pros*-positive cells in the focal plane shown here. (B) In *dri* mutant embryos, *pros* expression is absent in one cell per hemineuromere, depicted by an arrow. (C,D) Higher magnifications of A,B, respectively, showing that the four remaining *pros*-positive cells are not positioned in a flat sheet as they are in wild type. (E,F) *rC56*-directed β-galactosidase expression is reduced or absent in the longitudinal glia of *dri* mutant embryos (arrows in F).

DISCUSSION

To understand the mechanisms that control formation of the central nervous system, it is important to identify the genetic and molecular components involved. We provide evidence that the transcriptional regulatory gene, *dri*, is one such component, required for normal embryonic nervous system development. The primary evidence for this comes from the axon bundling and tracking defects observed in *dri* mutant embryos. The axon phenotype includes incorrectly bundled and routed connectives, and axons that sometimes join the wrong bundle or cross from one tract to another. However, the axon defects do not reflect a role for *dri* in neurons. *dri* is not expressed in the neurons that give rise to the longitudinal axon bundles, nor do these neurons exhibit a mutant phenotype in *dri* mutant embryos. Rather, *dri* was found to be expressed in a subset of glial cells, the longitudinal glia, A and B subperineural glia and the medialmost cell body glia, where it is required for normal differentiation and, as a consequence, normal axon fascicle formation.

Although *dri* is expressed early in glial development, two lines of evidence allow us to conclude that *dri* does not play a role in the neural/glial cell fate decision. First, ectopic expression studies showed that DRI cannot induce a glial cell fate in non-glial cells, nor was it involved in repression of the neural fate during glial cell formation. Second, loss of *dri* function was found not to impair the initiation of gliogenesis. The first axon tracts were established correctly, indicating that the *dri* mutation does not cause any dramatic glial cell fate changes. In addition, *dri* mutant longitudinal glia were still able to undergo their initial migration to the correct position relative to the midline and to navigate the growth cones of pioneer axons. This process depends on the ability of the Robo receptor, on the glial cell, to recognize the repulsive Slit signal emanating from the midline (Halter et al., 1995; Jacobs et al., 1989; Kinrade et al., 2001; Schmidt et al., 1997). Thus, we conclude that Robo function is not affected in *dri* mutants.

After the initial migration and pioneer axon navigation, however, the behaviour of *dri*-expressing glial cells becomes

aberrant. The normal final positions of these cells are never adopted and the cells exhibit cell shape defects. The mild misplacement of LG in *dri* mutants is probably caused by defects in glia-glia and axon-glia contacts, resulting at least in part from downregulation of the glial cell surface marker Neuroglian. These defects may interfere with correct migration of glia along the axon bundles which, in turn, causes the axon tract defects.

What is the molecular basis of the mutant phenotype found in *dri* mutants? DRI is a transcription factor, so the link between loss of *dri* function and the failure to differentiate properly is likely to be indirect, mediated through misregulation of *dri* targets required for normal longitudinal glial development. The most informative data came from our analysis of the position of *dri* in the glial transcriptional regulatory cascade. In general terms, *dri* activity was found to be downstream of *gcm* and *repo*, and independent of *pnt* and *cut*. It was also found to be upstream of two genes, *loco* and *pros*, which are essential for normal development of some glial cells. In this developmental context *dri* acts as an activator of downstream targets. A consensus DRI binding-sequence has been defined (Gregory et al., 1996), but neither the *loco* nor *pros* promoter regions contain such sequences. This raises the possibility that *loco* and *pros* might be indirect targets of DRI activity in the hierarchy of glial cell differentiation. Confirmation of this will require further analysis of *loco* and *pros* transcriptional regulation and perhaps the discovery of additional transcriptional regulatory factors involved in glial development.

The requirement for DRI in the activation of *loco* is unexpected. *loco* has been found to be a transcriptional target of PNT but not of REPO (Granderath et al., 2000; Granderath et al., 1999b), while we found that *dri* expression depends on REPO and not on PNT. It is possible that expression of *loco* is co-dependent on PNT and DRI in some cells and that the reduced level of *dri* expression observed in *repo* mutants is enough to permit *loco* expression.

The genetic analysis presented here strengthens the hypothesis that there are different genetic controls for different subsets of dorsal glia. For example, *dri* expression in all glial cells requires GCM activation, but only some of them requires REPO. The REPO-independent *dri*-positive cells, two per hemineuromere, appeared to correspond to the A and B subperineural glia (A/B SPG). These derive from neuroglioblast NB1.1 (Halter et al., 1995; Klambt and Goodman, 1991; Udolph et al., 1993), suggesting that REPO is required only for the expression of *dri* in cells derived from the lateral glioblasts. Unlike *dri*, *pnt* and its downstream target *loco* are not expressed in the MM CBG, which do not have a lateral glioblast origin. This suggests that there are different pathways of *pnt* and *dri* induction downstream of *gcm*.

At least some of these hierarchical transcriptional interactions may explain the phenotypes observed. The axon and mild positional defects of glia in *dri* mutants resemble phenotypes of other known late gliogenesis factors, such as those observed in *pnt*, *repo*, *loco* or *pros* embryos. It is known that early distribution of the glycoprotein Neuroglian is perturbed in *pros* mutant embryos (Jacobs, 1993). *loco* encodes a regulator of G-protein signalling (RGS) that has been shown to bind to a Gαi-subunit (Granderath et al., 1999b) and could regulate a G-protein signalling pathway involved in LG migratory behaviour. In addition, expression of Heartless, the *Drosophila* FGF receptor, in LG, and similarities between the *loco* and *heartless* mutant phenotypes (Granderath et al., 1999b; Shishido et al., 1997) leaves open the possibility that FGF could trigger final migration of glia along the longitudinal connectives. This hypothesis is strengthen by the recent finding that subcellular redistribution of Neuroglian from the plasma membrane to cytoplasm, which normally happens during final glial migration to enwrap axon bundles, is disrupted in *heartless* mutants (Takizawa and Hotta, 2001). Alternatively, it remains possible that additional targets of *dri* mediate the role of this gene in longitudinal glial differentiation.

Our studies add *dri* to the list of genes, including *pnt*, *repo*, *loco* and *pros*, that exhibit phenotypes that are much milder than those of the *gcm*, *glide2* and *Drop/Ltt* genes at the head of the dorsal glia hierarchy (Buescher and Chia, 1997; Campbell et al., 1994; Giesen et al., 1997; Granderath et al., 1999b; Halter et al., 1995; Kammerer and Giangrande, 2001; Klaes et al., 1994; Klambt, 1993; Klambt and Goodman, 1991; Xiong et al., 1994). It appears that diversification of these downstream regulators produces different types of glial cells. Nonetheless, each plays an essential role in driving the required behaviour of glial cells during CNS development. In the case of the DRI transcription factor, this role includes fine tuning the cell shape and migration characteristics of longitudinal glia that enable them to establish a normal axon scaffold.

This work was supported by the Australian Research Council. We are very grateful to Paul Whitington, Barry Dickson, Simon Koblar and William Chia for helpful discussions. We thank C. Goodman, P. Silver and A. Travers for providing antibodies. The monoclonal antibodies 9F8A9 anti-ELAV, 2B10 anti-CUT (developed by G.M.Rubin), MR1A anti-Prospero (developed by C.Q Doe), 22C10 (developed by S.Benzer), BP 102 anti-CNS axons (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.

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