Netrins guide migration of distinct glial cells in the *Drosophila* embryo

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**SUMMARY**

Development of the nervous system and establishment of complex neuronal networks require the concerted activity of different signalling events and guidance cues, which include Netrins and their receptors. In *Drosophila*, two Netrins are expressed during embryogenesis by cells of the ventral midline and serve as attractant or repellent cues for navigating axons. We asked whether glial cells, which are also motile, are guided by similar cues to axons, and analysed the influence of Netrins and their receptors on glial cell migration during embryonic development. We show that in Netrin mutants, two distinct populations of glial cells are affected: longitudinal glia (LG) fail to migrate medially in the early stages of neurogenesis, whereas distinct embryonic peripheral glia (ePG) do not properly migrate laterally into the periphery. We further show that early Netrin-dependent guidance of LG requires expression of the receptor Frazzled (Fra) already in the precursor cell. At these early stages, Netrins are not yet expressed by cells of the ventral midline and we provide evidence for a novel Netrin source within the neurogenic region that includes neuroblasts. Later in development, most ePG transiently express *uncoordinated 5* (*unc5*) during their migratory phase. In *unc5* mutants, however, two of these cells in particular exhibit defective migration and stall in, or close to, the central nervous system. Both phenotypes are reversible in cell-specific rescue experiments, indicating that Netrin-mediated signalling via Fra (in LG) or Unc5 (in ePG) is a cell-autonomous effect.

**KEY WORDS:** *Drosophila*, Frazzled, Longitudinal glia, Netrin, Peripheral glia, Uncoordinated 5

**INTRODUCTION**

During animal development, cells often change shape and position by morphogenetic movements as well as by active migration. Precise coordination of these processes during organogenesis is achieved by extensive cross-talk between motile cells and their environment. The most complex organ, the nervous system, is composed of a variety of neuronal cell types that become connected to each other and to non-neuronal cells in a highly specific manner to form functional networks. The accuracy with which these cells extend their neurites, read environmental signals and finally connect to their appropriate target sites needs to be controlled by an intricate molecular machinery (reviewed by Dickson, 2002; Grunwald and Klein, 2002; Huber et al., 2003; Chilton, 2006).

Among the factors involved are Netrins, secreted molecules that form a concentration gradient that is interpreted by navigating axons (reviewed by Livesey, 1999; Barallobre et al., 2005). Netrins were first discovered in vertebrates as neurotrophic morphogens that are secreted from the floor plate and attract neurons to cross the midline (Tessier-Lavigne et al., 1988; Pfaczek et al., 1990). The two vertebrate Netrins (netrin 1 and netrin 2) are highly homologous to the *Caenorhabditis elegans* Uncoordinated-6 (UNC-6) protein (Ishii et al., 1992), which was discovered together with UNC-5 and UNC-40 as guidance cues for pioneer axons (Hedgecock et al., 1990). It was shown that UNC-6 and the vertebrate Netrins primarily serve as diffusible ligands (Kennedy et al., 1994). UNC-5 and UNC-40 [the vertebrate homologues are Unc5 and deleted in colorectal carcinoma (Dcc), respectively] are Netrin receptors that promote either axon repulsion (UNC-5/Unc5) (Leung-Hagesteijn et al., 1992; Leonardo et al., 1997) or attraction (UNC-40/Dcc) (Chan et al., 1996; Keino-Masu et al., 1996).

In *Drosophila*, two Netrins (NetA and NetB) are described as guidance cues, e.g. for commissural and longitudinal axons and motoneurons (Harris et al., 1996; Mitchell et al., 1996; Labrador et al., 2005). Attraction of commissural axons towards the midline is mediated by the receptor Frazzled (Fra), the *Drosophila* homologue of UNC-40/Dcc (Kolodziej et al., 1996). Fra seems to guide commissural axons both by cell-autonomous signalling events (Forshoeelf et al., 2005; Brankatschk and Dickson, 2006; Garbe and Bashaw, 2007) and by capturing Netrin and presenting it to other neurons (Hiramoto et al., 2000). Repulsion of motoaxons away from the source of Netrin expression, however, is mediated via Unc5 (Keleman and Dickson, 2001; Labrador et al., 2005).

Both Netrins are secreted by cells of the ventral midline, a structure comparable to the vertebrate floor plate. Glial cells of the ventral midline not only secrete diffusible molecules that act as chemoattractants or repellents for navigating neurons (reviewed by Jacobs, 2000), but also serve as guidepost cells for axons projecting along or across the midline (Klämbt et al., 1991) (reviewed by Auld, 1999; Tear, 1999; Jacobs, 2000; Chotard and Salecker, 2004; Parker and Auld, 2004). Besides midline glia, other populations of glial cells in the developing nervous system interact with neurons. Longitudinal glia (LG), the embryonic interface glia, are involved in both the guidance of pioneer neurons and the fasciculation of longitudinal axon bundles (Hidalgo et al., 1995; Hidalgo and Booth, 2000) (reviewed by Auld, 1999; Parker and Auld, 2004; Parker and Auld, 2006). Glial cells in the transition zone between the central and peripheral...
nervous neurons (the CNS and PNS, respectively) guide both sensory neurons into the CNS and motoneurons out into the periphery (Sepp et al., 2000; Sepp et al., 2001; Parker and Auld, 2004).

All glial cells are themselves highly motile and migrate over considerable distances to occupy characteristic positions in the developing (embryonic) nervous system (reviewed by Parker and Auld, 2004; Klämbt, 2009). How do glial cells navigate? Do they use similar molecules and mechanisms to axons? In order to understand the mechanisms involved in glial cell migration and guidance, we analysed the influence of Netrins and their receptors on embryonic glial cells. We previously described the stereotypic migration of embryonic peripheral glia (ePG) as well as a collection of cell-specific markers to identify single, or subsets of, glial cells in the CNS and PNS of Drosophila (Beckervordersandforth et al., 2008; von Hilchen et al., 2008). This enabled us to analyse the effects of loss-of-function mutations of both Netrins and their receptors Unc5 and Fra in glial cells at single-cell resolution. Here we show that in Netrin mutants, two distinct populations of glial cells are affected. LG fail to migrate medially in the early stages of neurogenesis and we provide evidence that early Netrin-dependent LG guidance requires the expression of Fra already in the precursor cell, the longitudinal glioblast (LGB). By contrast, later in development, nearly all ePG transiently express unc5 (unc-5 – FlyBase) mRNA during their migratory phase. In unc5 mutants, however, two of these cells in particular show defective migration and stall at, or close to, their place of birth in the CNS. Both phenotypes are reversible by cell-specific rescue experiments, indicating that Netrin-mediated signalling via Fra (in LG) or Unc5 (in ePG) is a cell-autonomous effect. Furthermore, we demonstrate that the two Netrins are expressed within the neuroectoderm and in neuroblasts between stages 10 and 11 and can redundantly guide the LGB. Our results also suggest an exclusive role for NetB in mediating the repulsion of ePG away from the ventral midline.

MATERIALS AND METHODS
Fly stocks and genetics
The following fly strains were used: wild type OregonR; NetAΔ, NetBΔ, NetABΔ, NetAΔ-NetBΔpros™ (Brankatschk and Dickson, 2006); fraΔ and fraΔ’ [Bloomington Drosophila Stock Center (BDSC)]; unc5Δ (Labrador et al., 2005); simΔ (BDSC); cas-Gal4 (Hitier et al., 2001); elav-Gal4 (BDSC); gom-Gal4 combined with UAS-ncGFP (gift from A. Giangrande, IGBMC Strasbourg, France); Mz605-Gal4 and Mz1580-Gal4 (Ito et al., 1995); pros-Gal4 C21 (gift from F. Matsuzaki, CBD Kobe, Japan); repo-Gal4 (Sepp et al., 2001); sca-Gal4 (Klaes et al., 1994); sim-Gal4 (gift from C. Klämbt, University of Münster, Germany); UAS-NetA and UAS-NetB (Mitchell et al., 1996); UAS-fra (Kolodziej et al., 1996); UAS-HA-unc5 (Kelemen and Dickson, 2001); UAS-nlacZ (BDSC); UAS-Rho1.V14 and UAS-Rho1.N19 (BDSC). For labelling of distinct ePG and for rescue experiments the UAS-NetB system was used (Brand and Perrimon, 1993). Whenever required, lacZ- or GFP-tagged balancer chromosomes (BDSC) were used for identification of genotypes.

Immunohistochemistry
Embryos were fixed as described (Rogulja-Ortmann et al., 2007). Primary antibodies were mouse anti-Fas2 (1D4, 1:10) and mouse anti-Pro5 (1:5), both from DSHB (Iowa city, IA, USA); rabbit anti-Repo (1:500) (Halter et al., 1995); rabbit anti-Msh (1:500; gift from C. Doe, University of Oregon, USA); guinea pig anti-NeuG (1:500; chicken anti-β-gal (1:1000; Abcam, Cambridge, UK); and rabbit anti-Fra (1:500; gift from A. Stollewerk, University of London, UK). Cy3-, Cy5- or FITC-conjugated donkey secondary antibodies were used (1:250; Jackson ImmunoResearch, UK). For imaging, a Leica TCS SP2 confocal microscope was used. Images were processed using Leica Confocal Software and Adobe Photoshop.

In situ hybridisation
In vitro transcription and labelling of RNA were performed using the DIG-RNA labelling mix according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). In situ hybridisation in combination with anti-Repo antibody staining was performed as described (Altenheim et al., 2006). Images were taken with a Zeiss Axioskop 2 microscope and processed using AxioVision Software and Adobe Photoshop.

RESULTS
Netrins guide glial cell migration via Frazzled or Uncordinated 5
To investigate whether Netrins guide glial cell migration, we analysed mutants in which both Netrins are removed (NetABΔ) (Brankatschk and Dickson, 2006). Embryos at late stage 16 were stained for the glial marker Reversed polarity (Repo) and the neuronal cell adhesion molecule Fasciclin 2 (Fas2). We analysed the pattern of ePG in abdominal segments. Nearly all of the 12 ePG in each hemisegment (hs) have a unique identity, a stereotypic migration and an invariant final position at the end of embryogenesis (for details, see von Hilchen et al., 2008). In wild type, six of these cells finally align along the intersegmental and segmental nerves (ISN and SN, respectively) distal to the nerve branch of the SNC (Fig. 1A-A’, arrows). In NetABΔ mutants (Fig. 1B-B’), the number of ePG distal to the SNC was reduced in 94% of hs [NetABΔ, median of four cells (n=68 hs); wild type, median of six cells (n=100 hs); Fig. 1E]. Sometimes, more than three ePG were detected in the exit area of affected hs (between the SNCs and the CNS), suggesting that the ePG missing distal to the SNC stall in this area (Fig. 1B-B’, asterisk). This phenotype also occurred in NetBΔ single mutants at nearly the same penetrance, but with a slightly decreased expressivity (Fig. 1E). NetAΔ single mutants never showed this phenotype, indicating that ePG guidance is mediated by NetB, although the reduced expressivity in NetBΔ mutants compared with NetABΔ suggests a synergistic effect and hence some functional redundancy between the two Netrins.

In addition to the stalling phenotype of ePG, 29% of hs of NetABΔ mutants showed ectopic glial cell clusters in the dorsal periphery in close proximity to ePG11 (Fig. 1B-B’, curved arrow). Although all NetABΔ mutant embryos showed ectopic clusters, the number was variable, ranging from one to ten clusters per embryo. They were not obviously associated with neuronal structures. Analysis of the CNS of corresponding hs revealed a lack of cells dorsal to the longitudinal connectives, which are referred to as interface glia or LG. Analysis of Netrin single mutants revealed ectopic clusters with reduced penetrance and expressivity mainly in the posterior segments of NetAΔ mutants, but never in NetBΔ mutants (Fig. 1E). Furthermore, longitudinal axon tracts displayed fasciculation defects, especially in hs in which LG were missing (Fig. 1B). These longitudinal axon defects have been observed previously, but without reporting a glial phenotype (Harris et al., 1996; Mitchell et al., 1996).

We further investigated the two Netrin receptors Unc5 and Fra. In unc5Δ mutants, ePG stalling occurred with nearly identical expressivity as in NetABΔ, but we never found ectopic LG clusters (Fig. 1C,E); these were detectable only in fraΔ/fraΔ mutants in ~18% of hs (Fig. 1D,E, curved arrows). In addition to ectopic LG clusters, fraΔ/fraΔ mutants also showed a mild ePG stalling phenotype, with mainly one cell stalling in the exit area in 49% of hs (Fig. 1D,E).

The cluster phenotype is specific to LG
All LG are progeny of the LGB, which arises at the lateral edge of the neuroectoderm at stage 10. It successively divides while migrating towards the ventral midline, and gives rise to nine cells that align in
Netrins in glial cell migration

**Fig. 1. NetAB\(^{\Delta}\), unc5 and fra mutants exhibit different glial phenotypes.** (A-D\(\text{'}\)) Maximum projections of confocal stacks showing abdominal hemisegments (hs) (flat preparations) of late stage 16 Drosophila embryos of wild type (A,A\(\text{'}\)), NetAB\(^{\Delta}\) (B,B\(\text{'}\)), unc5\(^{\delta}\) (C), and fra\(^{f}/fra^{4}\) (D) stained for the glial marker Reversed polarity (Repo, magenta) and the neuronal cell adhesion molecule Fasciclin 2 (Fas2, green). Anterior is up. (A) Overview of two hs showing 12 ePG along peripheral nerves and 30 glial cells in the CNS per hs. (A\(\text{'}\)) Higher magnification of the boxed area in A. In each hs, six ePG (arrows) migrate from the CNS into the periphery and align along the SN and ISN distal to the SNC. (A\(\text{'}\)\(\text{'}\)) Schematic representation of the observed phenotypes in NetAB\(^{\Delta}\) mutants. (B) In NetAB\(^{\Delta}\) mutants, alterations of the glial pattern occur. Some ePG do not migrate into the periphery, resulting in fewer cells along the ISN and sometimes more than three ePG in the exit area between CNS and SNC (asterisk). Ectopic clusters of cells (curved arrows) occur in proximity to ePG11 (open arrow) and cells are missing in the CNS in corresponding hs (dotted circle). (B\(\text{'}\)) Higher magnification of boxed area in B. Four instead of six ePG (arrows) align along the ISN and SN distal to the SNC. (B\(\text{'}\)\(\text{'}\)) Schematic representation of the observed phenotypes in NetAB\(^{\Delta}\) mutants. (C) In unc5\(^{\delta}\) mutants, fewer ePG (arrows) are found along peripheral nerves distal to the SNC. Sometimes, one of these cells is located in the exit area (not shown). (C\(\text{'}\)) Schematic representation of the ePG phenotype in unc5\(^{\delta}\) mutants. (D) In fra\(^{f}/fra^{4}\) mutants, ectopic clusters of glial cells occur in the periphery (curved arrow). In corresponding hs, LG are missing in the CNS (dotted circle). In nearly 50% of hs, one ePG is missing distal to the SNC but stalls in the exit area (not shown). (D\(\text{'}\)) Schematic representation of the fra\(^{f}/fra^{4}\) phenotype. Ectopic clusters (curved arrow) occur in hs in which LG are missing in the CNS. The ePG stalling is indicated (asterisk). (E) Statistical evaluation of phenotypes in NetAB\(^{\Delta}\), NetA\(^{\Delta}\), NetB\(^{\Delta}\), unc5\(^{\delta}\) and fra\(^{f}/fra^{4}\) mutants (penetrance, percentage affected embryos; expressivity, percentage affected hs). Embryos were considered affected if demonstrating a phenotype in at least one hs. For expressivity, only affected embryos were considered. Dashed lines indicate the ventral midline. Dorsal sensory organ precursor-derived ePG are labelled with open symbols: ePG10, arrowhead; ePG11, arrow; ePG12, curved arrow. CG, channel glia; ePG, embryonic peripheral glia; ISN, intersegmental nerve; LG, longitudinal glia; M-ISNG, medial intersegmental nerve root glia; SNC, segmental nerve c.
rows dorsal to the longitudinal connectives (Jacobs et al., 1989; Schmidt et al., 1997) (reviewed by Auld, 1999; Parker and Auld, 2004; Stacey et al., 2007). In order to prove that ectopic clusters of glial cells in the periphery of NetAB, NetA and fra1/fra2 mutants indeed consist of LG, we stained for LG-specific markers. All LG can be labelled with an antibody against the Muscle segment homeobox (Msh; Drop – FlyBase) protein (Fig. 2A-B, yellow arrowheads) from stage 11 onwards (Shishido et al., 1997; Hidalgo et al., 2001; Beckervordersandforth et al., 2008). In addition, the medial intersegmental nerve root glia (M-ISNG) is Msh positive (Fig. 2B, yellow arrows) (Beckervordersandforth et al., 2008). In hs showing no ectopic LG, we detected nine plus one Msh-positive cells

Fig. 2. LG are ectopically located in NetAB. (A-H) Confocal stacks of two abdominal hs (flat preparations) of wild-type (A-D) and NetAB mutant (E-H) Drosophila embryos stained with anti-Repo (magenta) and various LG-specific markers (green). B,B′ F,F′ illustrate separations of dorsal stacks, whereas all other images show maximum projections. Anterior is up, dorsal to the left. Dashed lines indicate the ventral midline; symbols as in Fig. 1. (A) In wild type the LGB migrates medially from stage 11 onwards while dividing (resulting in two Msh-positive LG, yellow arrowheads). (B) At stage 16, Msh labels neurons (green) and ten glial cells (white nuclei). (B,B′) In a separation of dorsal stacks, the identity of the Msh- and Repo-positive LG (yellow arrowheads) and M-ISNG (yellow arrows) becomes more obvious. (C) All nine LG can be labelled with an antibody against the cytoplasmic protein Nazgul (Naz). (D) Six of these cells (the A- and M-clusters) also express the transcription factor Prospero (Pros). (E) In NetAB mutant the ectopic localisation of LG is observable already at stage 11 (shown with the marker Msh). In A3, LG are mispositioned (curved arrow), whereas in A4 the LG are located normally. (F) At stage 16, Msh labels LG and M-ISNG (white nuclei) both in the CNS and in LG within ectopic clusters (curved arrow). (F,F′) Separation of dorsal stacks facilitates the identification of LG (yellow arrowheads) and M-ISNG (yellow arrows). In A2 no LG are detectable in the CNS, but the M-ISNG is present. In A3, all nine LG and the M-ISNG are present. (G) The LG-specific marker Naz further validates that ectopic clusters consist of LG (curved arrow), which are absent in the CNS of corresponding hs (dotted circle). (H) Differential expression of Pros in six of the LG also occurs in ectopically located LG (curved arrow), again leaving gaps in the CNS of corresponding hs (dotted circles).
(LG and M-ISNG, n=100 hs) in the CNS (Fig. 2F-F’, hs A3). In the majority of affected hs (>75%), nine LG were detected within ectopic clusters in the PNS (Fig. 2F, curved arrow), while the Msh-positive M-ISNG was still present in the CNS (Fig. 2F’,F”, yellow arrows).

We also used an antibody directed against a protein, which we termed Nazgul (Naz), that is exclusively expressed in LG and cell body glia (CBG) (Fig. 2C). Naz is a cytoplasmic protein that is expressed in LG and CBG from stage 12 onwards until the end of embryogenesis (our unpublished data). Staining expressed in LG and CBG from stage 12 onwards until the end of embryogenesis (our unpublished data). Staining expressed in LG and CBG from stage 12 onwards until the end of embryogenesis (our unpublished data).

Having shown that most ectopic clusters consist of the entire LGB lineage, we next addressed whether expression of the transcription factor Prospero (Pros) also occurs within ectopic clusters. In wild-type stage 16 embryos, Pros is differentially expressed in six anterior LG (Fig. 2D) (Doe et al., 1991; Griffiths and Hidalgo, 2004; Beckervordersandforth et al., 2008). In NetABΔ mutants, on average six LG also expressed Pros (Fig. 2H) (although the standard deviation was higher than for the wild type). These data indicate that not only is cell number normal, but also that lineage-dependent cell specification of LG occurs normally in ectopic clusters and hence is independent of their normal position and postulated interactions with longitudinal axons (Griffiths et al., 2007). We also stained for the LG-specific markers in NetAΔ and fra1/fra2 mutants to confirm that the ectopic clusters in these mutants consist of LG (not shown).

Fra guides LGB migration cell-autonomously

The fact that the LG phenotype occurs already at early stages, immediately after the LGB has divided once, and that most ectopic clusters consist of all nine LG, strongly suggest that Netrin-Fra interaction is required already at late stage 10/early stage 11. We asked whether Fra acts cell-autonomously within the LG lineage. In agreement with previous data (Kolodziej et al., 1996), we could not detect any Fra protein in glial cells, including the LGB and its early progeny. Both anti-Fra antibody staining and in situ hybridisation revealed a broad fra expression domain lateral to the ventral neurogenic region at stage 10 (Fig. 3A). The LGB delaminates from this region, and in some cases we were able to detect fra mRNA within the Repo-positive LGB at early stage 11 (Fig. 3B). From these findings, we assume that Fra acts already in the LGB and becomes downregulated prior to, or immediately after, delamination. If such early Fra expression is required and sufficient to guide LG medially, it should be possible to rescue the cluster phenotype of fra1/fra2 mutants with an early glial-specific Gal4 driver. Indeed, gcm-Gal4-driven expression of UAS-fra in a fra mutant background was able to restore the phenotype (Fig. 3D and Table 1), whereas repo-Gal4-driven expression of UAS-fra could not (Table 1). We tested further Gal4 drivers that are known to be expressed in the LGB lineage, but only Mz1580 was able to weakly restore the LG phenotype (Table 1). Neuronal expression of UAS-fra (e.g. driven by elav-Gal4) did not rescue this phenotype at all (Table 1). Additionally, none of the glial Gal4 drivers rescued the mild ePG phenotype of fra mutants. These data indicate that Fra acts cell-autonomously within the LGB lineage, but has a non-autonomous effect on ePG migration. The stalling of mainly one ePG per hs in fra1/fra2 mutants is most likely a secondary consequence of pathfinding defects of peripheral axons.

The ventral midline is not responsible for LG migration

Fra is known to promote an attractive signal, e.g. for navigating neurons towards the ventral midline, where both Netrins are expressed. In order to test whether Netrins supplied by cells of the ventral midline are involved in the guidance of LG, we addressed whether the ventral midline is required for LG migration. In wild-type embryos, the LGB delaminates at the lateral edge of the neuroectoderm and in some cases a colabelling offra mRNA and Repo was detected (arrowhead), whereas fra expression is now detectable within cells of the developing nervous system. (C) gcm-Gal4-induced expression of UAS-fra (together with UAS-ncGFP) in a heterozygous fra/+ background does not alter glial migration (C), whereas in fra1/fra2 mutants it rescues the LG cluster phenotype (D) (see also Table 1). Occasionally, single LG are still ectopically positioned in the periphery (curved arrows). The weak ePG stalling phenotype of fra1/fra2 mutants cannot be rescued (arrows).

**Table 1. Experiments performed to rescue the LG phenotype in fra1/fra2 and NetABΔ mutants**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Driver line for rescue</th>
<th>Penetrance*</th>
<th>Expression*</th>
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<tr>
<td>fra1/fra2</td>
<td>–</td>
<td>100 (11/11)</td>
<td>18.2 (24/132)</td>
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<tr>
<td>fra1/fra2; UAS-fra</td>
<td>gcm-Gal4</td>
<td>52.4 (11/21)</td>
<td>10.0 (16/160)</td>
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<td></td>
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<td>23.4 (41/175)</td>
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<td></td>
<td>pros-Gal4</td>
<td>100 (16/16)</td>
<td>15.5 (22/213)</td>
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<tr>
<td></td>
<td>elav-Gal4</td>
<td>100 (22/22)</td>
<td>18.9 (56/296)</td>
</tr>
<tr>
<td>fra3/fra4</td>
<td>Mz1580-Gal4</td>
<td>86.7 (13/15)</td>
<td>19.6 (41/209)</td>
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<tr>
<td></td>
<td>Mz605-Gal4</td>
<td>100 (14/14)</td>
<td>20.0 (40/200)</td>
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<tr>
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<td>29.0 (61/213)</td>
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<tr>
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<td>11.8 (2/17)</td>
<td>10.0 (3/30)</td>
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<td></td>
<td>sim-Gal4</td>
<td>75.0 (9/12)</td>
<td>17.9 (24/134)</td>
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<tr>
<td>NetABΔ; UAS-NetB</td>
<td>sca-Gal4</td>
<td>8.3 (1/12)</td>
<td>6.7 (1/15)</td>
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<td></td>
<td>sim-Gal4</td>
<td>72.7 (8/11)</td>
<td>15.6 (19/122)</td>
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*Penetrance (%) is based on the existence of ectopic LG clusters (regardless of the number of clusters per embryo). The number of embryos demonstrating mispositioned LG is given in parentheses. Embryos displaying no, or only single, ectopic LG (up to four cells) in the periphery (see Fig. 3D, curved arrows) were considered as rescued.

1Expression (%) corresponds to the number of affected hs as shown in parentheses.
ventral midline are responsible for LG guidance, we used the sim-Gal4 driver to rescue the LG phenotype of NetAB<sup>4</sup> mutants. To our surprise, sim-Gal4-driven expression of either UAS-NetA or UAS-NetB only weakly restored the LG phenotype (Table 1). To further analyse the role of midline-derived Netrins in LG guidance, we investigated single minded (sim<sup>2</sup>) mutants. Sim is a key regulator of midline differentiation, and in sim<sup>2</sup> mutants midline cells are completely absent, leading to severe axon pathfinding defects and to a collapse of longitudinal axon tracts. Longitudinal glia, however, were positioned medially in sim<sup>2</sup> mutants and lay on top of collapsed longitudinal axons (Fig. 4B,D). Expression of the LG-specific markers Naz and Pros appeared unchanged in sim<sup>2</sup> mutants, as compared with wild type (Fig. 4A-D), but owing to the severity of the phenotype exact cell counts were not possible. We never found ectopic clusters of LG in the periphery of sim<sup>2</sup> mutants though, which we would expect if the midline served as a Netrin source for LG guidance.

Thus, we investigated the expression of both Netrins in early stages by mRNA in situ hybridisation (Fig. 4E-F). Between stages 9 and 11, Netrin expression is highly dynamic in different tissues, including mesoderm, neuroectoderm and neuroblasts (NBs). We used additional markers to define more precisely which NBs express Netrins (see Fig. S2 in the supplementary material). Some of these expression domains that included Netrin-expressing NBs lay medial to the site where the LGB delaminates (Fig. 4E-F, arrowheads) and could therefore potentially guide the LGB and its early progeny. We observed differences between NetA and NetB expression: in the posterior segments A5 to A8, NetA was predominantly expressed, whereas NetB expression was weak here. These differences in expression might explain why ectopic LG clusters occur in NetA<sup>4</sup> single mutants only in posterior segments, whereas NetB<sup>4</sup> single mutants never show ectopic clusters (Fig. 1E).

To prove that early Netrin expression within the ventral neurogenic region and in NBs serves as a cue for LG guidance, we expressed either UAS-NetA or UAS-NetB under the control of scabrous (sca)-Gal4, which drives expression broadly within the neuroectoderm from stage 8/9 onwards. In NetAB<sup>4</sup> mutants, sca-Gal4-driven expression of either Netrin resulted in complete rescue of the cluster phenotype in ~90% of embryos (Table 1). Ectopic expression of either Netrin with sca-Gal4 in an otherwise wild-type background also occasionally resulted in ectopic clusters. Why is it that sca-driven expression of Netrins restores the LG phenotype of NetAB<sup>4</sup>, but induces the same phenotype in an otherwise wild-type background? In the absence of endogenous Netrin, the sca-driven

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**Fig. 4. LG guidance depends on Netrin from the neuroectoderm but not the ventral midline.** (A-D) Confocal stacks of stage 16 *Drosophila* embryos (flat preparations) of wild type (A,C) and sim<sup>2</sup> mutants (B,D) stained with anti-Repo (magenta) and anti-Naz (green; A,B) or anti-Pros (green; C,D). Anterior is up. (A) The cytoplasmic protein Naz is expressed in LG that align along the longitudinal axons. (B) In sim<sup>2</sup> mutants the Naz staining validates that LG are still located on top of longitudinal axons even though the ventral midline is absent and the axon tracts are collapsed. (C) Pros is expressed in many neurons (green) and in six LG per hs (white nuclei). (D) In sim<sup>2</sup> mutants the differential expression of Pros within the LG lineage appears normal. (E-F) In situ hybridisation against NetA (E-E') or NetB (F-F') mRNA (blue) in combination with anti-Repo staining (brown) in flat preparations of stage 11 embryos. (E-E') In situ hybridisation against NetA mRNA at stage 11 shows a broad expression domain in the ventral neurogenic region medial to the LGB (arrowheads) throughout the entire embryo. (F-F') NetB is expressed medially to the LGB (arrowheads) and also in clusters of cells laterally. Expression becomes considerably weaker in posterior segments. For further details, see Fig. S2 in the supplementary material.
expression of either UAS-NetA or UAS-NetB is sufficient to significantly rescue LG guidance. However, in the presence of endogenous Netrin expression, additional Netrin expression by \textit{sca-Gal4} might overflow a medial-to-lateral gradient and hence abolish the directionality of the signal. This would support the hypothesis that Netrin-mediated signalling in LG serves as an instructive cue that provides directionality to LG migration, rather than serving as a permissive signal that provides motility per se without any direction. In order to prove this model, we ectopically expressed either Netrin in an otherwise wild-type background at early stages in the vicinity of the LGB, as induced by \textit{Krüppel-Gal4} within its gap-gene domain, or in a striped pattern in each segment, as induced by \textit{engrailed-Gal4}. All three drivers resulted in ectopic clusters of LG (with variable expressivity and penetrance; for details, see Fig. S3 in the supplementary material), which further supports the proposal that both Netrins act as instructive cues for LG migration.

Fig. 5. Most ePG transiently express unc5, but only distinct ePG stall in unc5\textsuperscript{8} mutants. (A,B) In situ hybridisation against unc5 mRNA (blue) in combination with anti-Repo staining (brown) in flat preparations of wild-type \textit{Drosophila} embryos at different developmental stages. (A) In wild type at early stage 15, all centrally derived ePG express unc5. (B) In late stage 16 wild-type embryos the in situ hybridisation signal fades. (C,D) Confocal stacks (flat preparations) of wild type (C) and unc5\textsuperscript{8} mutants (D,D'), each carrying the cas-Gal4>UAS-nlacZ transgene, stained with anti-Repo (magenta), anti-Fas2 (blue) and anti-\(\beta\)-gal (green). D' illustrates a separation of dorsal stacks, whereas all other images are maximum projections. Anterior is up and dorsal to the left. The CNS-derived ePG located distal to the SNC are indicated by arrows and the cas\(\triangleright\)nlacZ-labelled ePG6 and ePG8 are marked by arrowheads. Symbols as in Fig. 1. (C) ePG6 and ePG8 (arrowheads) are located distal to the SNC. (D) cas\(\triangleright\)nlacZ in an unc5\textsuperscript{8} mutant background shows a stalling of ePG6 and ePG8. In the anterior hs, one of the two cells lies in the exit area, whereas in the posterior hs, one cas\(\triangleright\)nlacZ-positive ePG has migrated into the periphery (arrowheads). In addition to the cas\(\triangleright\)nlacZ-positive ePG, occasionally ePG5 is missing too (asterisks). (D') Higher magnification of the boxed area in D. In both hs, the second (cas\(\triangleright\)nlacZ) labelled ePG is still located in the CNS (arrowheads). For further details, see Fig. S4 in the supplementary material. (E) Statistical evaluation of the number of cas\(\triangleright\)nlacZ-positive ePG (ePG6 and ePG8) located in the periphery (distal to the SNC), the exit area (proximal to the SNC) or in the CNS in unc5\textsuperscript{8} mutants as compared with wild type. For each genotype, at least nine embryos were evaluated. ISN, intersegmental nerve; Ich, lateral chordotonal organ; SNC, segmental nerve c.

Distinct ePG (ePG6 and ePG8) are guided by NetB and Unc5

It was previously reported that unc5 is expressed in ePG (Kelemen and Dickson, 2001; Freeman et al., 2003), although the resolution was insufficient to distinguish which. We observed transient expression of unc5 mRNA in ePG between stages 13 and 16 (Fig. 5A,B). But which ePG is affected in unc5\textsuperscript{8} mutants? With the capacity to identify nearly all ePG with molecular markers and by position at stage 16 (von Hilchen et al., 2008), we found that ePG6 and ePG8, which are progeny of NB2-5, were missing distal to the SNC in unc5\textsuperscript{8} and NetAB\textsuperscript{8} mutants. cas-Gal4>UAS-nlacZ was used to selectively label ePG6 and ePG8 (Fig. 5C, arrowheads) and confirmed that in homozygous unc5\textsuperscript{8} mutants, these two ePG either stall in the exit area proximal to the SNC (Fig. 5D, arrowheads) or remain in the CNS (Fig. 5D',E; see Fig. S4 in the supplementary material). Occasionally, ePG5 also stalled in the exit area (Fig. 5D,
We used an antibody (anti-SP2637) that is specific to a subset of ePG to further validate the selectivity of the observed phenotype in \textit{unc58} mutants (see Fig. S4 in the supplementary material).

In order to examine whether Unc5 function is required cell-autonomously in glial cells, rescue experiments were performed with either \textit{cas}-Gal4 or \textit{repo}-Gal4. \textit{cas}-Gal4-driven expression of \textit{unc5} in homozygous \textit{unc58} mutants led to significant rescue of the phenotype. The normal number of six ePG distal to the SNC was restored in 49% of hs (compared with 6% in \textit{unc58} mutants) and five ePG were found distal to the SNC in 39% of hs (compared with 24% in \textit{unc58} mutants) (Fig. 6C,F). Rescue experiments with \textit{repo}-Gal4 restricted the expression of UAS-\textit{unc5} to glial cells and also resulted in a significant rescue of the ePG stalling phenotype (Fig. 6D,F), with the normal number of six ePG distal to the SNC in 49% of hs (compared with only 6% in \textit{unc58} mutants) and five ePG distal to the SNC in 33% of hs (compared with 24% in \textit{unc58} mutants). Sometimes, one of the three proximal ePG (ePG1-3) migrated further distal, resulting in seven ePG lying distal to the SNC (11%, Fig. 5F).

Although pan-glial expression of \textit{unc5} in an otherwise wild-type background does not affect ePG migration, LG are often shifted to more lateral positions within the CNS (Freeman et al., 2003). We frequently observed two or more LG in the periphery [penetrance of 76.4% (\(n=17\) embryos) and expressivity of 19.5% (\(n=104\) hs)] and confirmed the identity of these ectopically located LG by anti-Naz staining (Fig. 6C,E, yellow arrowheads).

The finding that only \textit{NetB}\(^3\) single mutants show an ePG stalling phenotype and that Unc5 is required for ePG guidance suggest that ePG are repelled away from the CNS into the periphery [penetrance of 76.4% (\(n=17\) embryos) and expressivity of 19.5% (\(n=104\) hs)] and confirmed the identity of these ectopically located LG by anti-Naz staining (Fig. 6C,E, yellow arrowheads).

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Fig. 7. Midline-derived NetB guides ePG migration. (A-D) Confocal stacks of wild type (A), NetABΔ mutants carrying sim-Gal4 and either UAS-NetA (B) or UAS-NetB (C), and sim2 mutants (D). Flat preparations of stage 16 Drosophila embryos stained with anti-Repo (magenta) and anti-Fas2 (green). Anterior is up and dorsal to the right. Symbols as in Fig. 1. (A) In wild type, six centrally derived ePG are located distal to the SNC (arrows) and ectopic LG are never observed. (B) sim-Gal4-driven expression of UAS-NetA in midline cells of NetABΔ mutants is not sufficient to substantially rescue the stalling phenotype of ePG, resulting in a decreased number of ePG in most hs (arrows). Longitudinal tracts frequently collapse along the midline (arrowhead). The majority of embryos show ectopic LG in the periphery (curved arrow) (for statistics see Table 1). (C) sim-Gal4-driven expression of UAS-NetB in NetABΔ mutants can completely rescue the stalling phenotype of ePG in most hs, resulting in six centrally derived ePG distal to the SNC (arrows). Longitudinal tracts often collapse or abnormally cross the midline (arrowhead). Ectopic clusters of LG are found in ~75% of the embryos (curved arrow) (for statistics see Table 1). (D) In sim2 mutants the ventral midline is absent and the longitudinal axon tracts collapse. Peripheral nerves are also affected (asterisk). ePG show migration defects and stall in the CNS and exit area, resulting in fewer cells distal to the SNC (arrows). For further details, see Fig. S4 in the supplementary material. (E) Statistical evaluation of the sim-Gal4-driven rescue experiments with respect to ePG migration. For each genotype, at least ten embryos were evaluated.

DISCUSSION
A novel Netrin source is required for guidance, but not development, of the LGB lineage

Based on the present data, we postulate a dual role for Netrin-mediated signalling in glial cell migration. According to our model, early in neurogenesis, Netrins guide the LGB and its progeny from the lateral edge of the neuroectoderm towards a medial position. We show that at these early stages, Netrins are expressed by cells of the neuroectoderm as well as by NBs, and that this Netrin source most likely attracts the LGB via Fra. Ectopic expression of Netrins in the vicinity of the LGB might abolish a possible ventral-to-dorsal gradient and hence (occasionally) results in ectopic clusters (see Fig. S3 in the supplementary material). Additionally, we attempted to express Netrins in the dorsal area of the embryo and thereby attract the LGB and its progeny in the wrong direction. Unfortunately, none of the tested drivers showed Gal4 expression at the appropriate stage and intensity. Further experiments are needed to prove this model.

The LGB delaminates from the lateral neuroectoderm close to the sensory organ precursor-derived ePG11 (60–65% dorsoventral axis, where 0% is the ventral midline). In wild type, it migrates medially while proliferating, whereas we believe that in Netrin and fra mutants the LGB remains (and proliferates) at its place of birth and does not migrate at all in affected hs. Morphogenetic movements during germ band retraction, mesoderm migration and dorsal expansion of the epidermis complicate this issue. Nevertheless, ectopic clusters mainly remain in close proximity to ePG11. Although ectopic LG have no contact to axons, the lineage develops normally with respect to cell number and marker gene (Msh, Naz, Pros) expression. This is contrary to published data on the development and differentiation of LG, which have been postulated to depend on an intimate interaction with longitudinal axons (Griffiths and Hidalgo, 2004; Griffiths et al., 2007). Nevertheless, LG play an important role in the navigation and fasciculation of longitudinal axons (Hidalgo et al., 1995). Accordingly, in hs of Netrin and fra mutants, in which LG are mispositioned from the earliest stages, we found that the longitudinal axon tracts are thinner, show aberrant projections and fasciculation defects (see Fig. S5 in the supplementary material). These neuronal phenotypes were reported previously, but without noticing that the LG are missing in these hs (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Similar neuronal phenotypes can be induced by ectopic expression of unc5 in glial cells (repo>unc5) (see Fig. S5 in the supplementary material), which only affects LG and shifts them away from the midline to more lateral positions or even into the PNS. In some hs with ectopic LG clusters, longitudinal tracts show a weaker phenotype. In these cases, other glial cells (from within the same hs or an adjacent hs) fill the gaps on top of the longitudinal axons and hence seem to compensate for the loss of LG (data not shown; E. Giniger, personal communication).

From these data, we conclude that the longitudinal axon phenotypes observed in Netrin and fra mutants are, at least partially, a secondary effect of the lack of LG in corresponding neuromeres. Additionally (and somewhat confusingly), these neuronal phenotypes in fra mutants can be partially rescued by elav-Gal4 and
Mz605-Gal4 (Hiramoto et al., 2000; Keleman and Dickson, 2001), but neither rescues the LG phenotype (Table 1). Further experiments with other Gal4 drivers that allow a more restricted spatio-temporal expression of UAS-fra might help to resolve this issue.

**Distinct ePG are repelled away from the ventral midline by NetB**

The second population of glial cells that is guided by Netrin-mediated signalling comprises ePG. Nine ePG migrate from the ventral nerve cord into the PNS of each abdominal hs, but it is ePG6 and ePG8 in particular, both progeny of NB2-5, that show a stalling phenotype in NetABΔ, NetBΔ and unc5 mutants. We show by rescue experiments and analysis of Netrin single mutants that only NetB provides by cells of the ventral midline repels ePG6 and ePG8 via the Unc5 receptor. Although both Netrins are expressed by the ventral midline, they clearly do not share a redundant function for ePG guidance. A similar observation has been reported for unc5-expressing motoaxons, which respond differently to each Netrin (Mitchell et al., 1996; Winberg et al., 1998). To date, the nature of these differences between the two Netrins in combination with Unc5 remains unresolved. In NetAΔ-NetBΔ, only NetB is expressed, but is tethered to the membrane of the cell. In these embryos, no ePG stalling was observed (data not shown), further supporting the notion that only NetB is required for ePG migration and indicating that this signalling is at short range. But why do all ventrally derived ePG express unc5 mRNA transiently during their migratory phase? Further work will be needed to clarify why NetB-Unc5 signalling is selectively required for normal migration of ePG6 and ePG8.

**Are Netrins required for guidance of glial cells that do not migrate along axons?**

It is widely accepted that embryonic glial cells use neurons or neuronal processes as the substrate for migration (reviewed by Cafferty and Auld, 2007). It was shown previously that most migrating ePG follow certain axonal projections (reviewed by Silies et al., 2007). Could such neuron-glia contact be sufficient for proper guidance? The questions would then be (1) how do glial cells actually identify their respective neuronal projections and (2) how is directionality of migration given? Four-dimensional analysis of ePG migration, however, has revealed that ePG6 and ePG8 do not necessarily follow axons, but may also use other glial cells as substrate (von Hilchen et al., 2008). These two cells leave the CNS later than other ePG, they are the only ePG that can overtake other cells, and they may migrate on top of ePG rather than along peripheral nerves. Is it possible lack of axonal association (and perhaps adhesion) the reason why these cells need an additional guidance system?

The initial migration of the LGB and its early progeny cannot occur along axons because at these early stages axonal projections are not yet established. As discussed, in most cases the LG phenotype affects the entire LGB lineage and hence is an early guidance defect. So it might well be that early LG guidance is dependent on Netrin-Fra signalling without any neuronal contribution. After the first division of the LGB, neuronal projections are established, Net-Fra attraction is no longer required and fra expression is switched off. The results of our fra rescue experiments show that at least the timing of fra expression is crucial: gcm-Gal4-induced fra expression can rescue the LG phenotype, whereas a slightly later expression driven by repo-Gal4 cannot. The expressivity of the LG phenotype in NetABΔ mutants is only 30%. How are 70% of the LGB ‘rescued’? A second, as yet unknown, signalling mechanism could guide the LGB mediually, either by ventral attraction or dorsal repulsion. Similar redundancies have been reported, e.g. for border cell migration in the ovary or germ cell migration in early embryogenesis (reviewed by Ribeiro et al., 2003).

**Selectivity of Netrin-mediated signalling in glial cell migration**

In addition to the selectivity of the phenotypes in different populations of glial cells, as discussed above, another interesting observation comes from our rescue experiments in unc5 and fraΔ/fraΔ mutants. We performed control experiments to test whether pan-glial expression of either receptor affects glial cell migration. Glial expression of UAS-fra in an otherwise wild-type background does not alter the glial pattern in the CNS or PNS. Since unc5 is expressed normally in these experiments, repulsion of ePG6 and ePG8 into the periphery occurs as normal. By contrast, pan-glial expression of unc5 is able to shift LG to a more lateral position in the CNS [as reported previously (Freeman et al., 2003)] and LG can even leave the CNS and lie in the periphery, whereas all other glial cells are properly positioned. Why do only certain glial cells react upon Netrin-mediated signalling? More precisely, what conveys the ability for LG to ‘read’ Netrin-mediated signalling? In addition to the receptors, cells might require downstream molecules that could be differentially expressed and hence provide competence to react to Netrins. Several such molecules have been described for both vertebrates and invertebrates (Forsthoefel et al., 2005; Li et al., 2004; Li et al., 2006; Lucanica et al., 2006; Shekarabi et al., 2005). We have analysed loss-of-function mutants for Drosophila homologues of these possible downstream molecules, but none showed phenotypes comparable to those of fraΔ/fraΔ or unc5Δ (data not shown). Previous data demonstrate a function for the small GTPases Rac1 and Rho1 in ePG migration in Drosophila (Sepp and Auld, 2003), and it was recently shown that they can act downstream of Unc5 signalling in vertebrates (Picard et al., 2009). We expressed a dominant-negative form of Rho1 using cas-Gal4 in an otherwise wild-type background and obtained stalling of ePG6 and ePG8 (cas> Rho1N19, data not shown). Expression of a constitutively active form of Rho1 in ePG6 and ePG8 in an unc5 mutant background (unc5Δ; cas> Rho1V14), however, did not restore their stalling phenotype. Although we cannot rule out the possibility that ectopic expression of such constructs leads to artificial phenotypes, these data indicate that Rho1 is not downstream of Unc5, but rather acts in parallel. Further experiments will be needed to unravel the signalling complex of Unc5 and Fra/Dcc, and glial cell migration in the Drosophila embryo might serve as a powerful model system for this purpose.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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Netrins in glial cell migration


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