

# Commissure formation in the embryonic CNS of *Drosophila*

## II. Function of the different midline cells

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### SUMMARY

Most of the neurons of the ventral nerve cord send out long projecting axons which cross the midline. In the *Drosophila* central nervous system (CNS) cells of the midline give rise to neuronal and glial lineages with different functions during the establishment of the commissural pattern. Here we present evidence that beside the previously known NETRIN/FRAZZLED (DCC) signalling system an additional attractive system(s) is operating in the developing embryonic nervous system of *Drosophila*. Attractive cues appear to be provided by the midline neurons. We show that the glial cells present repulsive signals to the previously described ROUNDABOUT receptor in addition to a permissive contact-dependent

signal helping commissural growth cones across the midline. A novel repulsive component is encoded by the *karussell* gene. Furthermore the midline glial cells separate anterior and posterior commissures. By genetic criteria we demonstrate that some of the genes we have identified are acting in the midline glia whereas other genes are required in the midline neurons. The results lead to a detailed model relating different cellular functions to axonal patterning at the midline.

Key words: *Drosophila melanogaster*, Cell signalling, Commissural pattern, CNS, *karussell*, *roundabout*

### INTRODUCTION

The majority of neurons in the ventral nerve cord send their axons across the midline to the contralateral side. These neurons form the commissures in a close association with central nervous system (CNS) midline cells which in the vertebrate neural tube are called floor plate cells. Signals emanating from the midline cells organise the commissural axon pattern by either guiding growth cones towards the midline or by preventing them from crossing the midline (Tessier-Lavigne and Goodman, 1996).

Key components of the attractive signalling system orienting the commissural growth cones to navigate towards the midline cells are encoded by the *netrin* genes (Hedgecock et al., 1990; Kennedy et al., 1994; Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996). NETRINS can not only act as an attractive guidance cue but depending on the receptor present they can also mediate repulsive growth (Hamelin et al., 1993; Colamarino and Tessier-Lavigne, 1995). In addition, the response of the growth cone to a NETRIN gradient can be modulated by controlling the intracellular level of cAMP (Ming et al., 1997). The regulatory machinery needed to guide growth cones across the midline is likely to be much more complex. We have identified two additional genes which appear to be required for commissural axon tracts to cross the midline (Hummel et al., 1999).

Both attractive and repulsive cues are essential for the correct establishment of commissures in *Drosophila*. As contralateral projecting growth cones are guided towards the CNS midline, growth cones of ipsilateral projecting neurons must be hindered from crossing the midline. In addition, commissural growth cones are allowed to cross the midline only once. Part of the repulsive function is mediated by the *roundabout* (*robo*) gene product, first identified in *Drosophila* (Seeger et al., 1993). The ROBO protein is evolutionary conserved, but its ligand is unknown to date (Kidd et al., 1998a; Zallen et al., 1998). *roundabout* is expressed by lateral neurons, ROUNDABOUT protein distribution is controlled by the *commissureless* gene product which is expressed by a subset of CNS midline cells, the midline glia. Through a still unknown mechanism the COMMISSURELESS protein is transferred from the midline to axons of lateral neurons where it down regulates ROUNDABOUT expression in order to allow the commissural axons to cross (Tear et al., 1996; Kidd et al., 1998b).

The repulsive ligand of the ROBO receptor is likely to be expressed by the midline cells. In *Drosophila* the development of these cells is fairly well understood. In the developing ventral neural cord, 7-8 midline progenitor cells per abdominal segment generate about 26 glial and neuronal cells, i.e. 3-4 midline glial cells, 2 MP1 neurons, 6 VUM neurons, 2 UMI neurons, as well as the median neuroblast and its support cells

(Jacobs and Goodman, 1989; Klämbt et al., 1991; Bossing and Technau, 1994). The VUM neurons comprise motoneurons as well as interneurons, which project through the anterior and posterior commissures (Bossing and Technau, 1994). Genetic studies indicate that the VUM neurons are involved in the initial attraction of commissural growth cones (Klämbt et al., 1991; Hummel et al., 1998). The MP1 neurons are ipsilateral projecting interneurons, which participate in the formation of specific longitudinal axon pathways (Lin et al., 1995; Hidalgo and Brand, 1997). The median neuroblast divides during larval and pupal stages. Contrary to that which occurs in the grasshopper CNS the *Drosophila* median neuroblast does not generate midline glial cells (Condrón and Zinn, 1994).

In *Drosophila* the midline glial cells develop from a set of 2-3 progenitors located in the anterior part of each segment (Klämbt et al., 1991; Bossing and Technau, 1994). A function of the midline glial cells during the maturation of the segmental commissures has been found, such that two midline glial cells migrate along cell processes of the VUM-midline neurons to separate anterior and posterior axon commissures (Klämbt et al., 1991). If this migration is blocked a typical fused commissure phenotype develops. Towards the end of embryogenesis midline glial cells are required for the formation of individual fascicles within the commissures (Stollewerk and Klämbt, 1997).

Development of the midline glia is initiated by activation of the EGF-receptor (DER) encoded by *faint little ball* (Raz and Shilo, 1992). Conveyed through the conserved ras/MAPkinase pathway, DER activity is then translated into an activation of the ETS domain transcription factors POINTED and YAN (Scholz et al., 1997). POINTED subsequently controls the differentiation of the midline glial cells, and by activating the *argos* gene it triggers an inhibitory feedback loop in the midline, regulating the number of these cells (Scholz et al., 1997; Stemerink and Jacobs, 1997).

In a large scale EMS mutagenesis we attempted to identify most of the zygotically active genes required for the correct development of the axon pattern in the ventral cord of the *Drosophila* embryo (Hummel et al., 1999). Here we present a further analysis of the gene functions required during establishment of commissures in the ventral cord. In particular we focus on the role of midline glial cells in this process. Genetic analyses of mutations that affect commissural development revealed a long range repulsive function of the midline. We have identified mutations in two genes (*roundabout* and *karussell*) which appear to encode components of a contact-dependent repulsive signalling system. Additional genes which lead to a *pointed*-like phenotype have been identified and are likely to participate in controlling the migration of the midline glia (Hummel et al., 1999). Using genetic tools we show that at least one of these genes is required in the midline neurons for normal glial migration.

## MATERIALS AND METHODS

### Genetics

#### Identification of mutations affecting axon pattern formation

All mutations used in this work have been described by Hummel et al., 1999). Double mutant combinations were obtained following

standard recombination experiments or were generated using double blue balancer chromosomes (*CyO<sup>bb</sup>/Sp*; *TM6<sup>bb</sup>/TM3*). The *Df(2R)vg135* [49A-B; 49D-E] which removes the *frazzled* gene was obtained from the Bloomington *Drosophila* stock centre.

### Immunohistochemistry

Embryos were collected and stained as described previously (Hummel et al., 1997).

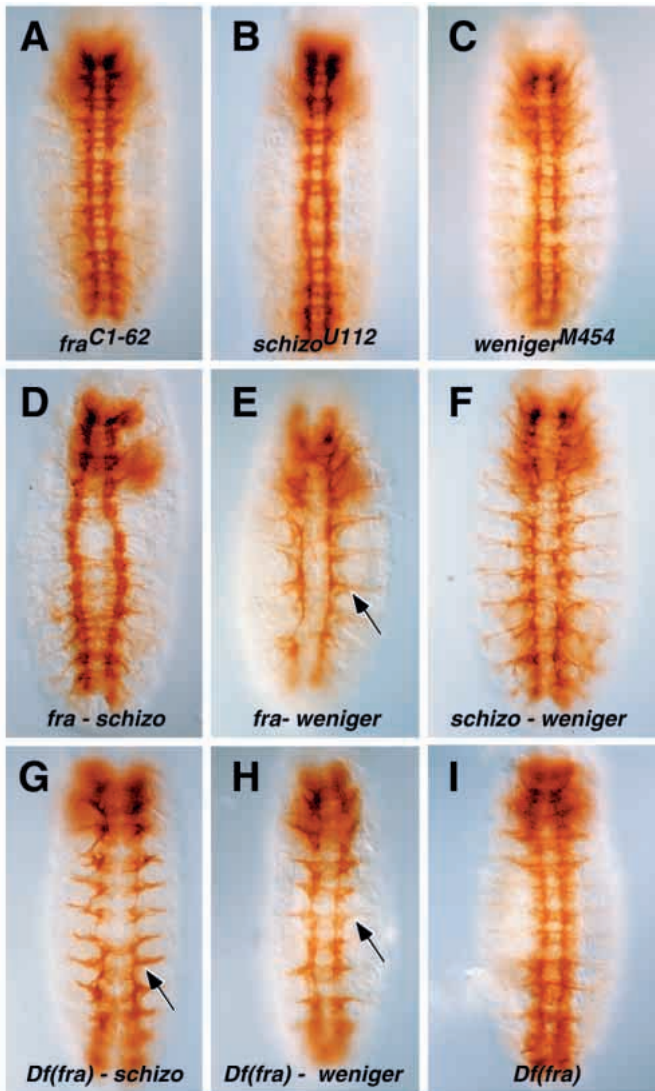
## RESULTS

The development of the wild-type ventral cord axon pattern has been described before (Klämbt et al., 1991; Goodman and Doe, 1993; for details see Fig. 1 in Hummel et al., 1999). In this accompanying paper we have described the identification of a number of gene functions which control different steps of commissure formation. In particular we have found 5 genes required for the initial establishment of commissures (*netrin*, *frazzled*, *schizo*, *weniger* and *commissureless*) and another 20 genes required for the separation of commissures. Here we focus on the functional interactions between these genes.

### Elimination of multiple attractive components reveals repulsive functions at the CNS midline

NETRIN and its receptor encoded by the *frazzled* gene are evolutionary conserved proteins which guide commissural growth cones across the midline (Kolodziej et al., 1995; Harris et al., 1996; Mitchell et al., 1996). However, even in the absence of any FRAZZLED or NETRIN protein some commissural fibres cross the midline (*frazzled* deficiency *Df(2R)vg135*, Fig. 1I; for *netrin* deficiency see Hummel et al., 1999; Fig. 5B). The remaining commissures detected in *netrin* or *frazzled* mutants could either be due to a NETRIN-independent guidance across the midline or these commissures could comprise axons which are normally repelled by a NETRIN signal. If a second attractive signal was required to guide commissural growth cones across the midline, the removal of both attractive signals should result in the loss of all commissural fibres. If axons cross the midline in *frazzled* or *netrin* mutants due to the repulsive action of the NETRIN protein, removal of additional attractive signals should not impede the formation of these commissural fibres in double mutant embryos.

In addition to the previously known genes we have identified another two genes which are required for the initial formation of commissures, *schizo* and *weniger* (Hummel et al., 1997). Based on their mutant phenotypes, the genes *schizo* and *weniger* are likely to encode either additional components of the NETRIN signalling system or define a second attractive guidance system. In order to obtain further insights in the function of these genes we generated several double mutant combinations (Fig. 1D-H). If *schizo* or *weniger* act downstream in the *netrin-frazzled* pathway, we would anticipate no enhancement of the commissural phenotype compared to the *frazzled* deficiency phenotype. In embryos homozygous for the hypomorphic allele *frazzled<sup>C1-62</sup>* (Fig. 1A) or the *schizo<sup>U112</sup>* allele (Fig. 1B) only some commissural connections are missing (2-4 neuromeres lack one commissure in every mutant *fra<sup>C1-62</sup>* embryo; 3-5 neuromeres lack one or both segmental commissures in every mutant *schizo<sup>U112</sup>* embryo, with >100 embryos analysed for each genotype). *weniger* mutant embryos



**Fig. 1.** Double mutant analyses suggest that a NETRIN-independent attractive and repulsive system operates at the CNS midline. Whole-mount antibody staining of stage 15 embryos using BP102 and subsequent HRP immunohistochemistry. Anterior is up. The different genotypes are indicated. The arrows in E, G and H point to CNS axons leaving the CNS. For further details see text. *Df(fra)* is *Df(2R)vg135* which removes the *frazzled* gene.

have a penetrant CNS phenotype and all neuromeres are affected. However, embryos double mutant for *frazzled*<sup>C1-62</sup> and *schizo*<sup>U112</sup> lack most commissural axons (more than 10 neuromeres per embryo lack commissures, with >30 embryos analysed for each genotype) (Fig. 1D). Similar synergistic effects are seen in *frazzled/weniger* or in *schizo/weniger* double mutant embryos (Fig. 1E,F). In addition to the commissural phenotype, we noticed an increased and pronounced growth of axons away from the CNS midline, especially in *Df(fra)/schizo* as well as in *fra/weniger* and *Df(fra)/weniger* mutant embryos (Fig. 1E,G,H arrows). The double mutant analysis also confirms the phenotypic differences found for *schizo* and *weniger*, with *weniger* showing a more severe phenotype. The *Df(fra)/schizo*<sup>U112</sup> phenotype is more severe compared to the

*fra*<sup>C1-62</sup>/*schizo*<sup>U112</sup> phenotype (Fig. 1D,G). A similar enhancement is not observed in *Df(fra)/weniger* embryos (Fig. 1E,H).

The double mutant phenotypes might suggest the presence of a long range repulsive signal originating from the midline, directing CNS axons out of the CNS via the segmental nerve (see below). We never observed a similar phenotype in embryos mutant for *schizo* or *weniger* or *frazzled* (Fig. 1B,C,I), nor in any other single mutation identified in the screen. Similar results were obtained when we analysed the *netrin* deficiency in several different double mutant combinations (data not shown). These double mutant analyses also indicate that axons crossing the midline in *fra* and *netrin* mutant embryos do not so because of a loss of a repulsive NETRIN signal.

In the light of the synergistic effect seen in the *frazzled/schizo* double mutant we suggest that beside the NETRIN and its receptor other proteins are required to guide commissural growth cones towards the midline. Furthermore, in the absence of two of the attractive signalling components, the existence of repulsive functions of the CNS midline is revealed. In the double mutant, the repulsive function predominates and directs axons out of the CNS.

### Repulsive functions of the midline

One receptor for the still elusive repulsive signal(s), presumably emanating from the midline, has been described recently as the ROBO gene product (Kidd et al., 1998a,b). In *robo* mutant embryos ipsilateral projecting growth cones, which normally do not cross the midline are able to project contralaterally (Seeger et al., 1993). Based on the phenotype we have identified 4 new *robo* alleles (Fig. 3A), which in all combinations showed partial complementation at 25°C, but were lethal at 29°C (no transheterozygous flies in >200 flies counted). Three *robo* alleles failed to complement the deficiency *Df(2R)X58-5M* which removes the *robo* gene (10% of the expected number of flies eclosed, >200 flies counted for each allele).

Beside *robo* (Fig. 3A) we have found one further complementation group which leads to a 'circling axon' phenotype. In stage 13 *karussell* (*kus*) mutant embryos, commissures appeared to be of uneven thickness when stained with a BP102 antibody. It appears that some commissural axons directly turn to cross the midline again, instead of projecting further laterally to grow along the forming longitudinal tracts (compare Fig. 2A,B, wild type to 2D-F, *karussell*; Fig. 2C shows a schematic drawing of the presumed axonal trajectories). We observed this phenotype in all mutant neuromeres. To analyse individual axon fascicles we stained *karussell* mutants with mAb 1D4 (anti FASII) and mAb 22C10. In wild-type embryos FASII protein is found on three longitudinal fascicles (Fig. 2I). In stage 12 *karussell* mutant embryos, several short FASII-positive cell processes project towards the CNS midline (not shown). In older embryos FASII expression is found on axons crossing the midline in more than 50% of the neuromeres (40 embryos analysed) (Fig. 2G,H arrows). The distribution of the 22C10 antigen in *karussell* embryos uncovered only few abnormalities (not shown). Thus a small subset of normally ipsilateral axons project contralateral in *karussell* mutants. The majority of axons found in the circles around the RP1 neurons are likely to be

commissural axons which cross the midline more than once. This suggests that *karussell* encodes a novel component of the repulsive signalling pathway.

In order to test whether *commissureless* interacts with *karussell*, as it does with *roundabout* (Kidd et al., 1998b), we generated the corresponding double mutant embryos. In *karussell/commissureless* double mutant embryos some axons can cross the midline but in most neuromeres the *commissureless* phenotype is epistatic to the *karussell* phenotype (Fig. 3E,D). However, if commissural axons have crossed the midline this neuromere shows a *karussell* phenotype. Thus *karussell* is either acting in parallel to *commissureless* and *roundabout* gene functions, or it acts downstream in a common regulatory hierarchy.

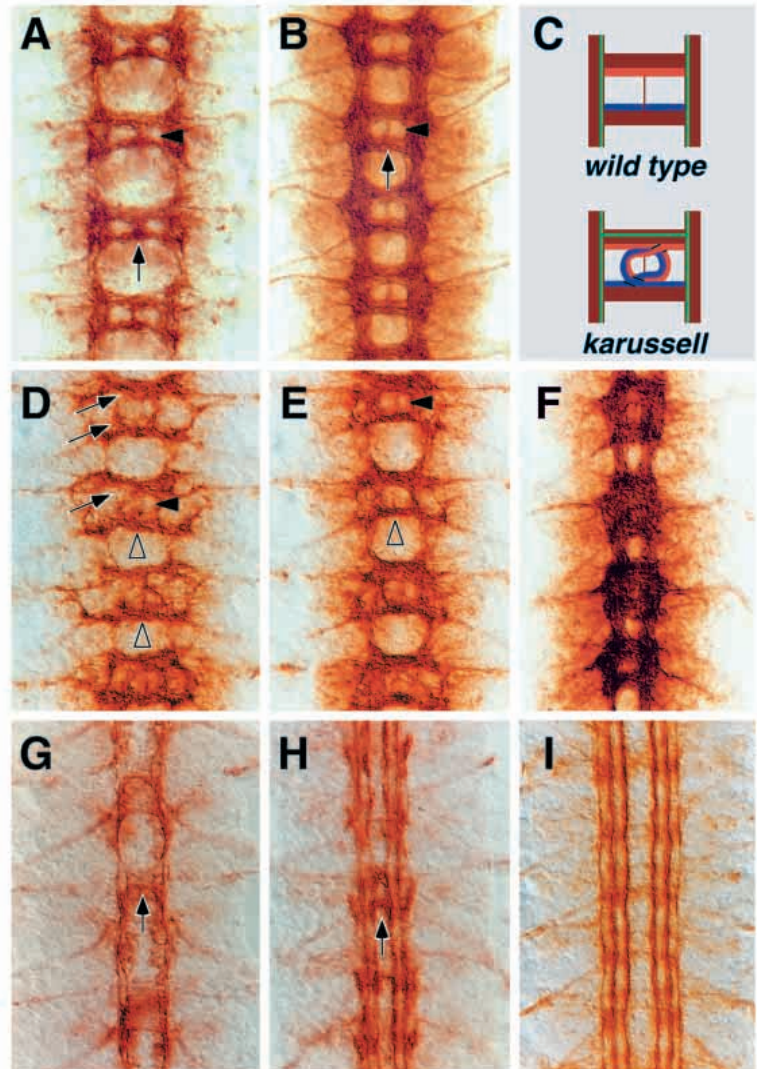
The expression of the repulsive receptor ROBO is regulated by the COMM protein (Kidd et al., 1998b). In the absence of COMM high levels of ROBO accumulate and subsequently no growth cones can cross the midline (Kidd et al., 1998b). To determine which CNS midline cells present the repulsive signal recognised by ROBO we have analysed double mutant embryos. In *commissureless* mutant embryos no commissures are formed (Fig. 3B). The gene *pointed* is specifically expressed in the midline glial cells and controls differentiation of this cell type (Klambt, 1993; Klaes et al., 1994). In *commissureless pointed* mutant embryos few commissures are formed (Menne, 1997). Similarly in *commissureless/slit* mutant embryos commissures do form (Fig. 3C). Since *pointed* as well as *slit* affect differentiation of the midline glial cells we suggest that these cells present the repulsive ligand to ROBO. In the absence of differentiated midline glial cells no repulsive ligand can be present and growth cones can cross the midline. This finding implies that disruption in glial differentiation at the midline should lead to a *robo*-like phenotype as well. Thus, we next examined the phenotype of mutants affecting the development of the midline glial cells. In *pointed* embryos we observed FASII positive axons crossing the midline (Fig. 3D). Besides *pointed* we identified other mutations in our screen which disrupt the differentiation of midline glial cells (Hummel et al., 1999). Some of these mutations also lead to a frequent crossing of FASII axons across the midline (*tramtrack*, Giesen et al., 1997; *cabrio*, *kästchen*, *klötzchen*, data not shown) whereas in *schmalspur* and *möchtegern* embryos we did not observe a crossing of FASII-positive axons (not shown).

### Further functions of midline glial cells

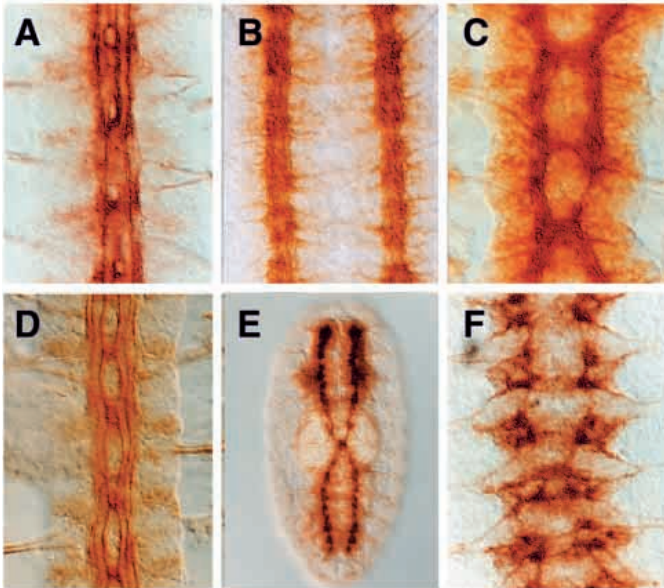
The formation of distinct anterior and posterior commissures requires intercalating migration of midline glial cells (Klambt et al., 1991). This migration depends on a neuron-glia interaction at the midline (see Fig. 6). Thus perturbation of midline glial migration could be either due to cell autonomous defects in the midline glia itself or could be due to defects in the interacting midline neurons. In our screen we identified many genes which appear to be required for midline glia migration and show fused commissure phenotypes upon mutation (Figs 4B, 5B-D). Based on additional phenotypic similarities,

12 of these genes have been placed in the so called *pointed* group (Hummel et al., 1999).

In order to define further functional relationships of the



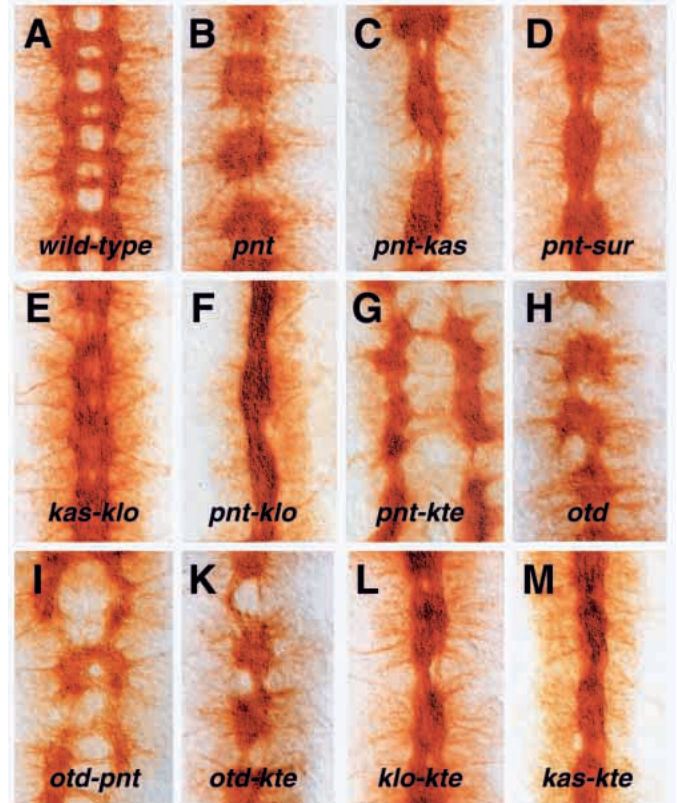
**Fig. 2.** The *karussell* phenotype. Frontal views of dissected central nervous system preparations. A-F were stained for the presence of all CNS axons using mAb BP102. G-I were stained for the presence of the FASCICLIN II antigen using mAb 1D4. Anterior is up. (A) In stage 13 embryos, separation of commissures has just started. (B) In stage 15 embryos, anterior and posterior commissures are well separated. In A and B the arrows point to processes of the VUM-midline neurons. Lateral to these processes reside the RP1 cell bodies (black arrowheads). (C) Schematic drawing of the wild-type (top) and the *karussell* (bottom) axon pattern phenotype. The red and blue lines indicate commissural fibers, the green line indicates longitudinal fibers. (D-E) Development of the CNS axon pattern in *karussell* mutant embryos. (D,E) In stage 13 embryos commissures have separated as in wild-type embryos, however the RP1 neurons (black arrowhead) appear closer to the midline. A conspicuous ring of axons surrounding the RP1 cell bodies can be detected. The commissures appear to be thicker at the midline (arrows). The open arrowheads point to processes of the VUM-midline neurons. Note, that the longitudinal connectives are not properly formed as in wild type (compare A and D). (F) In stage 15 embryos the circling axon phenotype is obscured as more and more axons cross the midline. (G,H) Mutant *karussell* embryos stained for the presence of the FASCICLIN II antigen. Some FASCICLIN II-positive axons cross the midline (arrows). (I) In wild-type embryos a crossing of FASCICLIN II-positive axons is never observed.



**Fig. 3.** Repulsive gene functions at the midline. (A-D,F) Frontal views of dissected stage 16 central nervous systems and (E) a whole-mount embryo stained for CNS axons. In A and D mAb 1D4, directed against the FASCICLIN II protein, was used; in the remaining panels mAb BP102 was used, which labels most CNS axons. (A-E) Stage 16 embryos, (F) stage 14 embryo. (A) In mutant *robo* embryos frequent crossing of FASCICLIN II-positive nerve bundles is observed. In contrast no FASCICLIN II-positive axons cross the midline in wild-type embryos (see Fig. 2I). (B) In mutant *commissureless* embryos no commissures can be detected. (C) In *commissureless/slit* double mutant embryos commissures are formed, however, in an irregular pattern. (D) In *pointed* mutant embryos, where the differentiation of CNS midline cells is disrupted, we observe frequent midline crossing of FASCICLIN II-positive axons, however not as extreme as in mutant *roundabout* embryos (A). (E) In *commissureless/karussell* embryos only occasionally do axons cross the midline. Interestingly, a *karussell* phenotype develops at the point of crossing the midline. The same epistatic relationship of the *commissureless* and the *karussell* phenotype can be observed in stage 13 embryos (F).

*pointed* group genes, we analysed a number of double mutant combinations. It has been shown before that *pointed* is expressed and required in the midline glial cells (Klämbt, 1993). We expected that embryos homozygous mutant for both *pointed* and other members of the *pointed* group would show a *pointed*-like CNS phenotype if both gene functions are required only in the glial cells.

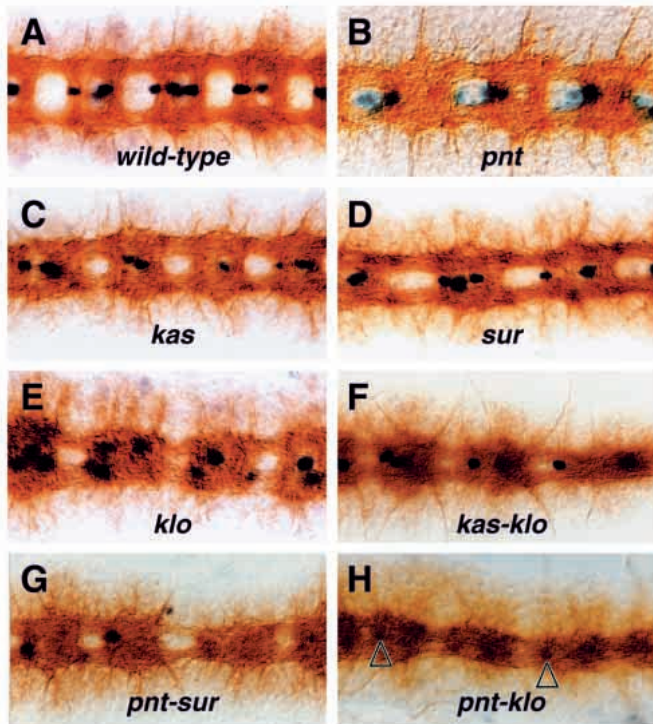
*pointed kästchen* or *pointed schmalspur* or *kästchen klötzchen* double mutant embryos show a slightly enhanced fused commissure phenotype as compared to the phenotypes of the single mutants (Fig. 4B-E). In *pointed klötzchen* double mutant embryos we observed a more severe fused commissure phenotype (Fig. 4F). In order to correlate these axonal phenotypes with the number and location of midline glial cells we analysed single and double mutant embryos with different enhancer trap markers (Fig. 5). In *pointed* embryos the midline glial cells fail to migrate at all (Fig. 5B). In contrast, partial midline glial cell migration combined with a slight reduction of the glial cell number, is observed in stage 16 *kästchen*, *schmalspur* and *klötzchen* embryos. In embryos double mutant for *kästchen klötzchen* or *pointed schmalspur* or *pointed*



**Fig. 4.** Double mutant analysis of *pointed* group mutations. Frontal views of dissected stage 16 central nervous systems. mAb BP102 and subsequent HRP immunohistochemistry was used to reveal the overall CNS axon pattern. Anterior is up. The genotype of the different single and double mutants is indicated. The mutant *kette* phenotype looks like the *pointed* mutant phenotype (Hummel et al., 1997). *pnt*: *pointed*; *kas*: *kästchen*; *sur*: *schmalspur*; *klo*: *klötzchen*; *kte*: *kette*; *otd*: *orthodenticle*.

*klötzchen* reduced numbers of midline glial cells are detected (Fig. 5F-H). This reduction corresponds well to the strength of the fusion of commissures. In the most extreme case only few glial cells are present per embryo (Fig. 5H, arrowhead). In addition these cells express the midline glial enhancer trap marker at low levels. Thus, we conclude that *klötzchen*, *kästchen* and *schmalspur* mainly function in the midline glial cells but compared to *pointed* have weaker effects on glial differentiation.

In contrast we observed a qualitatively different phenotype in *pointed kette* double mutant embryos (Fig. 4G). Here commissures failed to develop in most neuromeres, suggesting that *kette* does not function in the midline glial cells but rather might act in the midline neurons. To date the only other gene known to be required for the development of midline neurons is *orthodenticle* (*otd*), which encodes a transcription factor expressed in these cells (Finkelstein et al., 1990). In *otd* mutant embryos the posterior commissures fail to develop (Klämbt et al., 1991) (Fig. 4H). Interestingly, double mutant embryos for *otd* and *pointed* show a phenotype comparable to the *pointed kette* mutant (Fig. 4I,G). Furthermore, *otd*; *kette* double mutant embryos display an *otd*-like phenotype (Fig. 4K). This supports the notion that *kette* acts in the midline neurons as does



**Fig. 5.** The number of midline glial cells corresponds to the severity of the fused commissure phenotype. Frontal views of dissected stage 16 central nervous systems stained for the presence of the BP102 antigen and  $\beta$ -galactosidase expression in the midline glial cells driven by the enhancer trap insertions *AA142* (A-D,F,G) and *rD59* (E,H). The genotypes are indicated. The number of midline glial cells is reduced in the different double mutant embryos. Arrowheads in H point to a few remaining midline glial cells expressing the *rD59* midline glia marker. The expression of the *rD59* marker in *pointed* mutant embryos (not shown) is comparable to that in E. *pnt*: *pointed*; *kas*: *kästchen*; *sur*: *schmalspur*; *klo*: *klötzchen*.

*orthodenticle*. The failure of midline glial cell migration in *kette* mutant embryos would then be a non cell autonomous consequence of a defect in the midline neurons (see Discussion for further details).

In summary our genetic analyses show that *pointed*, *kästchen*, *schmalspur* and *klötzchen* act in the midline glia, whereas *kette* acts in the midline neurons. Only when we disrupted the development of neurons and glial cells in the midline as in *otd pointed* or *pointed kette* double mutant embryos did no commissures develop. However, in *klötzchen kette* or *kästchen kette* double mutant embryos we found a fused commissure phenotype (Fig. 4L,M). This supports our previous notion that mutations in *klötzchen* or *kästchen* have weaker effects on midline glia development than does *pointed*.

## DISCUSSION

In the preceding paper (Hummel, 1999) we have described the identification of genes needed for commissure development. Here we concentrated on the analysis of those genes that are required for the attraction of commissural growth cones and the subsequent separation of commissural tracts (summarized in Fig. 6).

### Attractive functions

In a saturating genetic approach we have identified mutations in three genes which are required to guide commissural growth cones towards the midline (*frazzled*, *schizo*, *weniger*) (Kolodziej et al., 1996; Hummel et al., 1999). The newly identified genes could either act in the same genetic pathway as the redundant *netrin* genes (Harris et al., 1996; Mitchell et al., 1996) or they could encode components of independently acting signalling systems. An example is the recently described Eph related RTKs, *Sek4* and *Nuk*, which cooperate in guidance of commissural axons in the vertebrate brain (Orioli et al., 1996).

Mutant animals that lack the *netrin* gene(s) show a reduction in the number of commissural fibres crossing the midline (Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996). Depending on the NETRIN receptor or the intracellular cAMP level, NETRIN can either act as an attractive or a repulsive signal (Serafini et al., 1996; Leonardo et al., 1997; Ming et al., 1997). In *Drosophila*, two *netrin* genes are found in close proximity. Only the deficiency removing both *netrin* genes leads to a reduction in the number of commissural axons crossing the midline. However, neither deletion of both *netrin* genes nor the removal of the NETRIN receptor encoded by the gene *frazzled* leads to a complete loss of commissural tracts (Kolodziej et al., 1995; Harris et al., 1996; Mitchell et al., 1996). It thus appears likely that additional components guide growth cones towards the midline.

Several new gene functions implied in NETRIN signalling have recently been identified in *C. elegans* that suppress an axon guidance phenotype produced by ectopic expression of *unc-5*, which encodes a NETRIN receptor (Ishii et al., 1992; Chan et al., 1996; Colavita and Culotti, 1998; Colavita et al., 1998). However, *weniger* and *schizo* are likely to act independent of the NETRIN signal as suggested by the synergistic effects seen in *frazzled/netrin* and *weniger* or *schizo* double mutant embryos. Thus *weniger* and *schizo* appear to encode new components guiding commissural growth cones towards the midline.

A NETRIN synergizing activity (NSA) was identified during its initial biochemical purification which potentiates the function of NETRIN in the neurite outgrowth assay (Serafini et al., 1994). It is unlikely that *schizo* or *weniger* encode such a component, because both genes produce a commissural phenotype which would not necessarily be expected for the NSA. We have now initiated a molecular analysis of *schizo* and *weniger* to reveal the nature of these newly identified gene functions and their role during commissure formation.

### Repulsive functions

In embryos double mutant for *schizo* and *frazzled* apparently all attractive components are removed thus disclosing repulsive signals originating from the midline. An evolutionary conserved repulsive receptor has been recently described as the *robo* gene product (Kidd et al., 1998a). The ROBO protein is expressed by lateral neurones and prevents axons from crossing the midline (Kidd et al., 1998a). Given the severe CNS phenotype associated with *robo* mutations it is surprising, that all *robo* alleles showed partial complementation at room temperature but nevertheless show a penetrant CNS phenotype under these conditions. A possible explanation would be intragenic complementation suggesting that ROBO acts as a multimer.

We identified one X-chromosomal gene, *karussell*, which shares some phenotypic traits with *robo*. However, based on the analysis with the mAb BP102 we suggest that *karussell* is primarily required to prevent commissural growth cones from recrossing the midline. ROBO protein distribution is regulated by *commissureless* which is expressed by the midline glial cells from where it is transferred to crossing axons (Tear et al., 1996; Kidd et al., 1998b). *comm/robo* double mutant embryos show a *robo* phenotype but *comm/kus* double mutant embryos show a *commissureless* phenotype, indicating that *kus* is independent or downstream to the *comm/robo* signalling process. This is expected if KUS normally prevents the recrossing of commissural axons and ROBO prevents crossing of longitudinal axons. Alternatively *kus* could be required to upregulate *robo* expression in commissural axons that have just crossed the midline.

### Function of the midline neurons

Attractive and repulsive signal originating from the midline are required for normal commissure development. The *Drosophila* midline comprises glial and neuronal cell lineages. Our data indicate that these two cell types exert distinct functions during commissure formation.

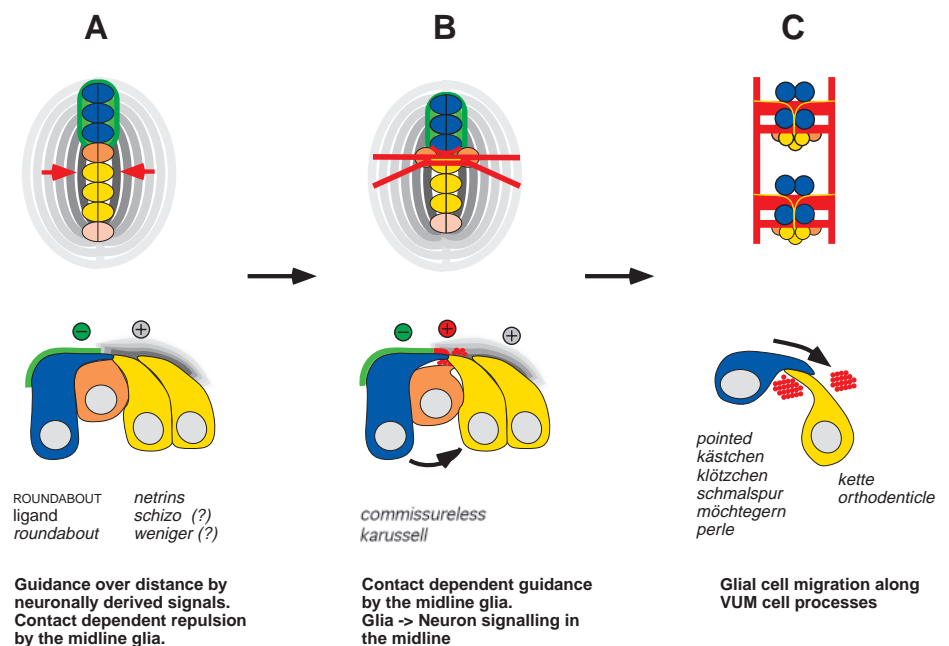
First commissural growth cones invariably steer towards the anterior-most VUM neurons where they cross the midline to form the posterior commissure. This indicates that initially the midline neurons attract the commissural growth cones. The *netrin* genes which encode an attractive signal for commissural growth cones are expressed in midline neurons and glial cells during initial commissure formation (Harris et al., 1996; Mitchell et al., 1996). However, the number of commissural fibres is normal in mutations affecting the development of the midline glia. Similarly, ablation experiments using the directed expression of *reaper* and *grim* in the midline glial cells resulted in a fused commissure phenotype and did not lead to a reduction in the number of commissural axons crossing the midline (Zhou et al., 1995). Thus, we propose that the midline glial cells do not play an essential role in attracting the commissural growth cones. The glial derived NETRIN signal could be required to counteract repulsive signals (see below).

Additional support for the assumption that the midline neurons attract commissural growth cones is provided by the *orthodenticle* mutant phenotype. Here some midline neurons as well as one of the two segmental commissures is missing (Finkelstein et

al., 1990; Klämbt et al., 1991). Similarly, expression of dominant negative JUN in all midline cells resulted in a loss of midline neurons and a concomitant loss of all commissures (Scholz et al., 1997). Furthermore, in *patched* mutant embryos the midline glial cells are almost absent and appear to be transformed into midline neurons (Hummel et al., 1999). Attraction of commissural growth cones is normal in these embryos, however commissural axons stall at the midline. This suggests that the midline glial cells do not participate in attracting commissural growth cones but provide locally acting, contact dependent cues helping growth cones across the midline (indicated in bright red in Fig. 6). Similarly, in the vertebrate neural tube changes in growth cone morphology have suggested that commissural axons are guided by a contact dependent mechanism across the floor plate (Bovolenta and Dodd, 1990; Stoeckli et al., 1997).

### Function of the midline glial cells

Our genetic data suggest, that in addition to being a permissive substrate for commissural growth, the midline glial cells



**Fig. 6.** Schematic summary of the different steps leading to commissure formation in the *Drosophila* embryo. (A) early stage 12, (B) late stage 12, (C) stage 15, embryos. The top panel shows frontal views of one segment, the bottom panel shows lateral views of tangential sections at the midline. The midline glial cells are indicated in blue, the MP1 neurons are indicated in orange, the VUM-neurons are indicated in yellow and the median neuroblast is indicated in pale orange. Axons are shown in red. (A) Signals emanating from the midline neurons first attract commissural growth cones. This attractive guidance signal comprises NETRIN and components of the SCHIZO/WENIGER system (grey). Concomitantly the midline glia express the ROUNDABOUT ligand and/or the *karussell* gene product (green) to prevent crossing of ipsilateral projecting axons and recrossing of axons which have already crossed the midline once. (B) In addition to the long range acting NETRIN molecules the midline glial cells express molecules that allow a contact-dependent crossing of the midline (indicated in bright red). The action of the attractive and repulsive signal directs the commissural axons to cross the midline at the interface of VUM-neurons and midline glia. The MP1 neurons move away from the midline and are thus unlikely to participate during commissure development. (C) At stage 12 neuron-glia interaction starts which finally results in an intercalating migration of the midline glial cells inbetween anterior and posterior commissure. Genes required in the different steps are indicated.

present the repulsive signal to axons which should not cross the midline. Indeed, we found many examples of FASCICLIN II-positive axons crossing the midline in mutants in which the development of the midline glial cells is affected (Fig. 3) (Giesen et al., 1997). The same defect can be observed when differentiation of the midline glia is impaired by directed overexpression of *argos*, which is a negative regulator of the EGF-receptor pathway (Stemerink and Jacobs, 1997). This finding was not unexpected since the COMMISSURELESS protein which regulates ROBO expression is found in high levels in these cells.

Thus we propose that one important function of the midline glial cells is to act as a control post dictating who can cross and who not. They prevent commissural axons from crossing the midline more than once and ensure that ipsilateral projecting axons never cross the midline. These two processes might be regulated by different processes (*karussell/roundabout*).

### Separation of commissures

Beside guiding commissural axons, the midline glial cells are required for the shaping of the two segmental commissures (Klämbt et al., 1991; Stollewerk and Klämbt, 1997). The formation of two distinct segmental commissures in each segment requires an intercalating migration of two of the 4-6 midline glial cells. This migration is preceded by a neuron-glia interaction at the midline. A surprisingly high number of genes appear to be required for this process (Hummel et al., 1998).

Axons that cross the midline have to differentiate between the dorsal and ventral side of the migrating midline glial cells/extending VUM neurons to be routed into the appropriate commissure (see Fig. 6). Thus, some mutations which lead to a fused commissure phenotype could stem from glial cells defects where the dorsal and ventral sides cannot be distinguished any more. Subsequently anterior commissure neurons would grow on the dorsal side of the CNS next to the posterior commissure. However, following migration, the midline glial cells would then be located below the segmental commissures.

Some of the mutations will not affect glial migration in the first place but will rather control the general differentiation of midline glial cells as for example *pointed* does (Klämbt, 1993; Klaes et al., 1994). Since neuronal migration along glial processes in the vertebrate system depends on several receptor systems (Fishman and Hatten, 1993) it appears possible that we have identified some components required specifically for glial migration along neuronal processes, which also occurs in the visual system of *Drosophila* (Choi and Benzer, 1994).

Based on the double mutant analysis at least one of the genes we have identified, *kette*, appears to be required in the midline neurons. Indeed, we recently isolated a P-element induced *kette* mutation which shows a specific  $\beta$ -galactosidase expression in the VUM-neurons, suggesting that *kette* is expressed in neuronal midline cells (Hummel, unpublished data). Here *kette* could either control neuronal development or could be involved in the actual neuron-glia interaction at the midline.

But why do commissures fail to develop in *pointed kette* double mutant embryos? If our assumption is correct, that attraction of first commissural growth cones is mediated by signals emanating from the midline neurons, this would imply that neuronal differentiation at the midline is more defective in

the double mutant compared to both single mutants. As a consequence neuronal differentiation as well as glial differentiation in the midline must depend on *pointed* function. In the CNS *pointed* is expressed only in glial cells and in *pointed* mutant embryos the VUM neurons are present and appear to project in their normal pattern. However, they fail to properly differentiate and do not express *orthodenticle* at high levels (data not shown). Whether this disruption of VUM glia differentiation is due to a lack of *pointed* in the midline glia or depends on *pointed* function in the VUM support glial cells is presently unknown. Based on this we can also deduce that *klötzchen* and *kästchen* do not influence the development of the midline neurons but encode new components regulating glial development.

### Conclusions

Following an extensive mutagenesis we have identified a distinct set of zygotically active genes required to set up the commissural axon pattern (Hummel et al., 1999). Here we have linked gene functions required for commissure formation to neurons and glial cells at the ventral midline of the CNS. We propose the following model as summarized in Fig. 6. The initial growth of commissural growth cones towards the midline in stage 12 embryos is guided by an attractive signal expressed by the midline neurons (Fig. 6A). Presumably, this attraction is mediated by early NETRIN expression in the midline neurons or alternatively by the action of a SCHIZO/WENIGER attractive system. At this early developmental stage the midline glial cells are elongated in shape contacting the epidermis with their basal side (Scholz et al., 1997) and are assumed to send out cellular processes contacting the VUM-midline neurons at the dorsal side of the nervous system. The midline glial cells express a repulsive signal which is conveyed to lateral axons via the ROBO receptor and/or the *karussell* gene product. This repulsive function restricts the first axons to cross the midline just anterior of the VUM neurons. The midline glial cells also express a contact dependent permissive guidance cue helping the axons to cross the midline (Fig. 6B, indicated in red). Subsequently neuron-glia interaction at the midline results in the migration of the midline glial cells along processes of the VUM neurons.

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