Determination of cell fate along the anteroposterior axis of the Drosophila ventral midline

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The Drosophila ventral midline has proven to be a useful model for understanding the function of central organizers during neurogenesis. The midline is similar to the vertebrate floor plate, in that it plays an essential role in cell fate determination in the lateral CNS and also, later, in axon pathfinding. Despite the importance of the midline, the specification of midline cell fates is still not well understood. Here, we show that most midline cells are determined not at the precursor cell stage, but as daughter cells. After the precursors divide, a combination of repression by Wingless and activation by Hedgehog induces expression of the proneural gene lethal of scute in the most anterior midline daughter cells of the neighbouring posterior segment. Hedgehog and Lethal of scute activate Engrailed in these anterior cells. Engrailed-positive midline cells develop into ventral unpaired median (VUM) neurons and the median neuroblast (MNB). Engrailed-negative midline cells develop into unpaired median interneurons (UMI), MP1 interneurons and midline glia.

KEY WORDS: Cell fate determination, Ventral midline, Embryonic CNS, Drosophila, Engrailed, Hedgehog, Wingless

INTRODUCTION

The ventral midline in Drosophila and the floor plate in vertebrates are specialised tissues that serve as central organisers during CNS development. During early embryogenesis, the floor plate and the ventral midline secrete signals that determine cell fates in adjacent tissues (reviewed by Arendt and Nubler-Jung, 1999). In vertebrates, Sonic hedgehog is secreted by floor plate cells, and controls cell fate in the adjacent somites and motoneuron cell fate in the neural tube (Echelard et al., 1993; Ericson et al., 1996; Hynes et al., 1995; Roelink et al., 1994; Teillet et al., 1998). In Drosophila, a TGFβ homologue called Spitz is secreted by the ventral midline and directs ventral cell fates in the ectoderm, mesoderm and developing CNS (Buescher et al., 1998; Gabay et al., 1996; Golembo et al., 1996; Luer et al., 1997; Schweitzer et al., 1995; Skeath, 1998; Yagi et al., 1998). In later embryogenesis, the floor plate and the ventral midline are essential for establishing a functional axon network. The two tissues secrete proteins of the widely conserved Netrin and Slit multitudes (reviewed by Araujo and Tear, 2003; Strahle et al., 1998). Expression of the first subset-specific gene on the midline is only detected at stage 10, about one hour after the midline precursors first divide. At this stage, the pair-rule gene odd skipped (odd) becomes restricted to the MP1 interneurons (Ward and Coulter, 2000). However, the mechanism by which subsets of midline cells acquire their different fates is still unknown.

Because all midline cells have the same dorsoventral positional information, it is likely that genes conferring anteroposterior information control midline cell fate. Using Engrailed expression as a reference, we identified the anteroposterior origin and the gene expression patterns of midline subsets at stage 10. In the midline, Engrailed is expressed in two distinct periods. Early Engrailed expression in the midline starts at the blastoderm stage and its maintenance in midline daughter cells depends on Wingless signalling. At stage 10, repression by Wingless and activation by Hedgehog restricts Lethal of scute expression to the most anterior midline daughter cells of the neighbouring posterior segment. Subsequently, Hedgehog induces Engrailed expression in all cells of the Lethal of scute cluster. In spite of their anterior origin, late Engrailed-expressing midline cells join the adjacent anterior segment and develop into posterior midline cells, VUM and MNB neurons. Ectopic expression of Hedgehog is sufficient to induce ectopic Engrailed expression in anterior midline cells, and to...
suppress the differentiation of MP1 interneurons and midline glia. Our data indicate that an early step in midline cell determination is the separation of midline siblings into non-Engrailed- and Engrailed-expressing compartments by the opposing functions of Wingless and Hedgehog signalling.

MATERIALS AND METHODS

Drosophila stocks

The following Drosophila lines were used: Oregon R (for wild type), wg^{1-17}/CyO (Baker, 1988), wg^{4-5}/wg^{12-12} (Besiovec and Martinez Arias, 1991), hh^{7/TM3sib} (Mohler, 1988) and hh^{71} (Ma et al., 1993), hh^{71} is a strong temperature-sensitive allele. Homozygous flies were kept at the permissive temperature (16°C) and embryos were shifted to the restrictive temperature (29°C) at different stages of development. The developmental stage of each embryo was determined by inspection prior to each temperature shift.

We used the following GAL4 lines: sca-GAL4 (Klaes et al., 1994), which drives expression in the neuroectoderm, CNS and, transiently, in the midline (stage 10-13), with expression gradually decreasing from stage 13; V2H-GAL4 (kindly provided by D. St Johnston) (Haeccker and Perrimon, 1998), which drives expression of Hedgegog and Wingless ubiquitously from cellular blastoderm until early stage 11, and then in scattered cell clusters in the epidermis; sim-GAL4/CyO; sim-GAL4/sim-GAL4 (Scholz et al., 1997), which we use expression in all midline cells from stage 10 to stage 13 and is gradually restricted to midline glia, although tau-GFP and β-galactosidase perdure in all midline cells throughout embryogenesis; and en-GAL4 (A.H.B., K. B. Yoffe and N. Perrimon, unpublished) (Fietz et al., 1995), to drive UAS-tau-GFP expression (Brand, 1998).

The UAS lines are: UAS-en (Yoffe et al., 1995), UAS-hh^{hh4} (Fietz et al., 1995) and UAS-wg^{ex}. Embryos expressing UAS-wg^{ex} were raised at the permissive temperature, 18°C. Hedgehog signalling was blocked by expression of UAS-Ci76 (Azal-Blanc et al., 1997). To follow cell morphology, we expressed UAS-tau-lacZ (Hidalgo et al., 1995) and UAS-tau-GFP (Brand, 1998).

The following crosses were used to study the differentiation of midline cells after loss, or ectopic expression, of Hedgehog and Wingless: UAS-hh/sim-GAL4;UAS-tau-GFP/sim-GAL4, sim-GAL4/UAS-tau-GFP, hh^{71}, sim-GAL4/UAS-hh, hh^{71}, UAS-tau-GFP/sim-GAL4, UAS-wg^{ex}/sim-GAL4, wg^{12-17}, sim-GAL4/UAS-tau-GFP and wg^{12-17}. UAS-hh/sim-GAL4/sim-GAL4. Mutant embryos were selected by the loss of Hedgehog expression, of Wingless expression, or, in the case of wg^{ex} embryos, of Engrailed expression.

Cell transplantation, Dil-labelling and immunohistochemistry

Cell transplantation was performed as described previously (Technau and Campos-Ortega, 1986). yw; V2H-GAL4/UAS-hh and yw; V2H-GAL4/++; UAS-wg^{ex}/+; embryos were used as donors. Donors were labelled by injection of 1% lysine-conjugated Texas Red dextran (70,000 M	extsubscript{r}; Molecular Probes) and 5% HRP in 0.2 M KCl. Cells were removed along the ventral midline from four to five segments: the donor cells come from different anteroposterior positions but the same dorsoventral position. Up to six donor cells were implanted into the same host segment and placed in a dorsoventral position comparable to their origin. Donors and hosts were both at stage 8. Midline precursors were labelled with Dil about 10 minutes after gastrulation. The anteroposterior position of the two midline siblings was documented about two hours (stage 10) and three hours (stage 11) after labelling. Dil labelling, photoconversion and immunohistochemistry were performed as previously described (Bosson and Technau, 1994; Bossing et al., 1996).

Antibodies were diluted in PBT (0.3% Triton in PBS) and newborn calf serum (20%) as follows: rabbit anti-β-galactosidase, 1:1000 (Cappel); mAbBP102, 1:50 (kindly provided by N. Patel); Seeger et al., 1993; rabbit anti-Cl, 1:50 (M. Fietz, unpublished; kindly provided by M. van den Heuvel and P. Ingham); mouse anti-Futsch (mAbBP102), 1:3 (kindly provided by M. Bate and S. Benzer) (Fujita et al., 1982); mouse anti-En, 1:2, and rat anti-Gsbd, 1:3 (kindly provided by R. Holmgren); rabbit anti-Hh, 1:2000 (A. Taylor, unpublished; kindly provided by M. van den Heuvel and P. Ingham); mouse anti-Imp, 1:10 (kindly provided by N. Patel) (Patel et al., 1989); rabbit anti-Odd, 1:1000 (Ward and Coulter, 2000); mouse anti-Ptc, 1:250 (kindly provided by I. Guerrero) (Capevilla et al., 1994); mouse anti-Slit, 1:10 (kindly provided by D. Hartley) (Rothberg et al., 1988); mouse anti-Wg, 1:10 (Strigini and Cohen, 2000).

Secondary antibodies conjugated to alkaline phosphatase, biotin, HRP (Jackson Laboratories), Alexa488 or Alexa568 (Molecular Probes) were used at a dilution of 1:250 in PBT (0.3% Triton in PBS) and newborn calf serum (20%). Biotin-coupled antibody reactions were enhanced using the Vectastain ABC Kit (Vector Laboratories).

All embryos were mounted as flat preparations in 90% glycerol in PBS. Images were taken using a Zeiss axiophot with DIC optics or a BioRad MRC 1024 confocal scan head on a Nikon E800 microscope. Images were assembled in Adobe Photoshop.

RESULTS

The anteroposterior position of midline lineages at stage 10

The determination of midline cells appears to take place during germband elongation, as by germband retraction most midline subsets can be identified by the expression of unique molecules (Jacobs, 2000). We decided, therefore, to identify the anteroposterior position of midline siblings during germband elongation. We labelled midline precursors with the lipophilic dye DiD or DiI (Molecular Probes) in embryos expressing GFP in the Engrailed domain (en-GAL4/UAS-tau-GFP). After division of the precursors, we followed the daughter cells throughout development, recording their segmental position at stage 10 and stage 11 (Fig. 1). MP1 interneurons (n=4, Fig. 1D,E), UMI (n=5, Fig. 1F,G) and MNB neurons (n=9, Fig. 1K,L) each arise from one precursor, and their daughter cells occupy fixed anteroposterior positions during germband elongation. The four daughter cells of the two glial precursors can be located either in the middle of the segment (n=2, Fig. 1A) or just anterior to the Engrailed domain (n=3, Fig. 1B). VUM neurons (Fig. 1J) arise from three midline precursors, and the six daughter cells of these precursors are located inside the Engrailed domain (n=3, Fig. 1H) and immediately posterior to the domain, in the anterior of the next segment (n=9, Fig. 1G).

In summary, we show that the midline glia and MP1 interneurons are the most anterior midline subsets, followed by a second pair of midline glia and a pair of UMIIs, and, finally, the VUM and MNB neurons. DiI labelling cannot resolve whether the MP1 interneurons or the midline glia are the most anterior cells. As determination of the MP1 interneurons depends on Notch/Delta signalling (Spana and Doe, 1996), it is possible that the anteroposterior position of the most anterior midline cells, the midline glia and MP1 interneurons, is random. Interestingly, four VUM neurons and the MNB neurons seem to arise from the anterior compartment of the next posterior segment. These cells initiate Engrailed expression half-way through germband elongation, and, during germband retraction, they join the adjacent anterior segment to become the most posterior midline subsets.

A molecular map of midline cells at stage 10

To identify molecules involved in the determination of midline cells, we combined the results from our in vivo studies with an expression analysis of midline cells throughout development. This approach allowed us to deduce a subset-specific expression for the different midline cell types (Fig. 2G). Surprisingly, Engrailed is expressed in midline cells in two phases (Fig. 2A). The early phase of Engrailed expression (early Engrailed) starts with two midline precursors. After the division of the two precursors, the number of Engrailed-positive midline siblings is reduced to about two cells at stage 10 (Fig. 2A-D). Early Engrailed-positive cells, also express the segmentation gene Gooseberry distal (Fig. 2B). During mid stage
nucleus enables cells to be counted. Ventral views, anterior to the left. The two midline siblings is in focus. Note that the absence of DiI from the posterior to it (I). The three other subsets of midline cells show a anterior to the Engrailed domain (B). (H,I) At stage 11, ventral unpaired midline glia are located either in the middle of the segment (A) or anterior to the Engrailed domain (B). (H,I) At stage 11, ventral unpaired median interneurons are positioned within the Engrailed domain (H), or posterior to it (I). The three other subsets of midline cells show a reproducible anteroposterior position. In panel A and K only one of the two midline siblings is in focus. Note that the absence of Di from the nucleus enables cells to be counted. Ventral views, anterior to the left. 10, the late phase of Engrailed expression (late Engrailed) starts in six midline cells positioned immediately posterior to the early Engrailed cells (Fig. 2A-D). Engrailed continues to be expressed in VUM interneurons (Siegl and Jia, 1999) and MNB neurons (Patel et al., 1989). The onset of late Engrailed expression is preceded by the expression of the proneural gene lethal of scute (Fig. 2C,D), and for about one hour expression of Engrailed and Lethal of scute coincide (until early stage 11). Interestingly, Lethal of scute-expressing cells (Fig. 2E) and cells expressing late Engrailed (data not shown) show strong expression of the Hedgehog receptor Patched, as revealed by the expression of the lacZ gene controlled by the patched enhancer element (Fig. 2E), or by anti-Patched (data not shown). The expression of Patched in Lethal of scute- and Engrailed-positive midline cells suggests that these midline cells receive the Hedgehog signal (see below).

Our in vivo analysis indicated that midline cells expressing late Engrailed appear to be part of the anterior half of the adjacent posterior segment. To confirm their anterior identity, we examined the expression of odd skipped (odd), a segmentation gene expressed in stripes in the anterior of each segment (Ward and Coulter, 2000). Unfortunately, Odd expression disappears from the midline with the onset of lethal of scute expression. Yet, the six Odd-positive midline cells, like the six Lethal of scute-expressing cells, also express Patched (Fig. 2F). In summary, a late phase of Engrailed expression is initiated in midline cells of the anterior half of the next posterior segment.

**Hedgehog induces the expression of Lethal of scute and late Engrailed in midline cells**

Cells receiving the Hedgehog signal activate Patched, the Hedgehog receptor (Chen and Struhl, 1996; Goodrich et al., 1996). Patched is upregulated in midline cells expressing Lethal of scute and late Engrailed, suggesting that Hedgehog may control the expression of both genes. In hedgehog mutants, Lethal of scute expression is lost in midline cells, but not in the adjacent CNS (Fig. 3A,B); early Engrailed expression is unaffected but late Engrailed is never induced (Fig. 3C,D). In wild-type embryos at stage 10, the number of Engrailed-positive cells increases from two to eight per segment, but hedgehog mutants have zero to five, with most segments having only two Engrailed midline cells. At the end of embryogenesis, wild-type embryos have six to nine Engrailed-positive midline cells per segment, whereas hedgehog mutants have between zero and three.

In the adjacent ectoderm, hedgehog is essential for the maintenance of wingless expression, and Wingless signalling activates Engrailed (Heemskerk et al., 1991). To establish that Hedgehog, and not Wingless, controls the expression of late Engrailed at the midline, we blocked Hedgehog signalling in midline cells by expressing a truncated version of the Hedgehog signal transducer Cubitus interruptus (Ci76). The Ci76 truncation mimics the short form of Cubitus interruptus, which is able to repress Hedgehog, but not Wingless, target genes (Aza-Blanc et al., 1997). Midline targeted expression of Ci76 from stage 10 leads to a reduction in Engrailed expression during germband retraction (Fig. 3E). Furthermore, restoring Hedgehog expression from stage 10 in midline cells of hedgehog mutants (sim-GAL4/UAS-hh; hh) is sufficient to activate Engrailed (Fig. 3F). Surprisingly, not all midline cells express UAS-hedgehog in a hedgehog mutant background. To activate expression in early midline cells, we used a fly strain carrying a fusion between the single-minded (sim) promoter and GAL4 (Brand and Perrimon, 1993; Scholz et al., 1997). In hedgehog mutants, Sim expression disappears from most midline cells during stage 10 (see Fig. S1 in the supplementary material). The downregulation of Sim in hedgehog mutants may also affect the sim promoter element driving GAL4, and thereby reduce the number of cells producing the GAL4 activator.

Our experiments suggest that Hedgehog, and not Wingless, controls late Engrailed expression in midline cells. Hedgehog is expressed in midline cells until mid stage 11 (see Fig. S2A in the supplementary material), after which time Hedgehog continues to be expressed in the adjacent neuroectoderm and CNS (see Fig. S2B in the supplementary material). To determine the time period during which Hedgehog is required to induce Engrailed expression, we
inactivated Hedgehog at different time points during embryogenesis using a temperature-sensitive hedgehog allele. When Hedgehog is inactivated during stage 10 and stage 11, the number of midline cells expressing Engrailed at stage 17 is reduced to zero to three cells, the same number as in hedgehog mutants (see Fig. S2C in the supplementary material). Even when Hedgehog is inactivated at late stage 11, about 30 minutes after the end of midline expression of Hedgehog, the number of Engrailed-expressing cells is reduced to about four cells in most segments. Later inactivation has no effect on Engrailed expression in the midline (Fig. S1C). The temperature shifts indicate that Hedgehog expressed in midline cells, as well as neuroectodermal Hedgehog, controls the expression of late Engrailed in the midline.

We labelled all midline cells with GFP to examine their differentiation in hedgehog mutant embryos (Fig. 3G,H). Midline cells are not incorporated into the CNS, but are positioned along the dorsal surface of the nerve cord. The number of midline cells per segment is severely reduced and the remaining cells stay undifferentiated. The reduction of midline cells may be partially due to the loss of Sim expression in hedgehog mutants. Yet, the increased number of GFP-labelled cell fragments indicates that many midline cells die in hedgehog mutants (Fig. 3H).

Because the expression of Lethal of scute precedes the expression of late Engrailed, we also examined whether Engrailed expression depends on lethal of scute. We followed Engrailed expression in the deficiency Tp1;2sc19, which removes the proneural genes achaete, scute and lethal of scute. This deficiency can be used as a lethal of scute mutant, because achaete and scute are not expressed in midline cells (Skeath and Carroll, 1992). Consistent with earlier reports (Martin-Bermudo et al., 1995), we confirm that the loss of lethal of scute causes a severe reduction in midline Engrailed expression (Fig. S1I). The expression of early Engrailed is not affected. At the end of stage 10, instead of about the eight cells in wild type, only four to six midline cells in every segment express Engrailed. Engrailed expression in the midline of Tp1;2sc19 embryos declines further during germband retraction. Ectopic expression of Lethal of scute in all midline cells is not sufficient to activate Engrailed (data not shown).

In summary, Hedgehog is needed to activate Lethal of scute expression in midline cells. From stage 10 to stage 12, Hedgehog and Lethal of scute are essential to induce and maintain late Engrailed expression in the midline. In hedgehog mutants, many midline cells die and surviving midline cells are undifferentiated.

**Wingless represses lethal of scute expression**

Because lethal of scute is essential for the expression of late Engrailed in midline cells, we assayed whether Wingless signalling controls Lethal of scute expression. In wild-type embryos, about six midline cells per segment form the Lethal of scute cluster (Fig. 4A). In wingless mutants, the number of Lethal of scute-positive midline cells per segment increases to eight to ten cells per segment. This increase suggested that in wild type, Wingless signalling represses Lethal of scute at the midline. The earliest time we can express Wingless in all midline cells is stage 10. At this stage, ectopic...
Wingless no longer interferes with midline cell differentiation (Fig. 6L). Therefore, we decided to test whether Wingless represses Lethal of scute by removing neuroectodermal cells from embryos ubiquitously expressing Wingless (V2H-GAL4/+; UAS-wg+/+) and implanting them next to the midline of wild-type embryos. Host embryos were examined for Lethal of scute expression at stage 10, using the expression of Gooseberry-distal or Wingless as a segmental marker. All Wingless-expressing cells that integrate outside the endogenous Wingless domain (n=3) repress the expression of Lethal of scute at the midline (Fig. 4C). Most of the transplanted cells integrate into the Wingless domain (n=8) and therefore do not become sources of ectopic Wingless. These cells do not affect Lethal of scute expression. Cells transplanted as a control between wild-type embryos never interfere with the expression of Lethal of scute.

Fig. 3. Expression of Lethal of scute and late Engrailed in midline cells depends on Hedgehog. (A) At stage 10, midline cells posterior to the Engrailed domain (En, brown) initiate Lethal of scute (L'sc, black) expression. (B) In hedgehog mutants, expression of Engrailed weakens, and Lethal of scute expression in the midline is absent. (C) At stage 13, Engrailed-positive midline cells (black) form a dense cluster. (D) In hedgehog mutants, the number of Engrailed-expressing midline cells is severely reduced. (E) The expression of a 76 kDa truncation of the signal transducer Cubitus interruptus (Ci76) blocks the reception of the Hedgehog signal. Blocking Hedgehog reception in all midline cells leads to a severe reduction in Engrailed-expressing midline cells. (F) The ectopic expression of Hedgehog (HH, brown) in midline cells of hedgehog mutants is sufficient to activate Engrailed (black). Expression of Engrailed in the midline is limited to Hedgehog-positive cells but not all midline cells express Hedgehog. (G) Engrailed (red) is still expressed in a subset of midline cells in a stage 17 embryo. The axons of midline cells are labelled by expression of tau-GFP (green). (H) In hedgehog mutants, midline cells (yellow) are not incorporated into the CNS. Midline cells do not extend axons and the number of midline cells is severely reduced. Only a few Engrailed-positive midline cells (red) survive. Arrows indicate cell fragments. (I) The loss of lethal of scute in the mutant Tp(1;2)sc19 results in a reduction in Engrailed-positive midline cells, which is comparable to hedgehog mutants. Ventral views, anterior to the left; brackets outline the midline.

Fig. 4. Wingless signalling opposes Hedgehog signalling in the regulation of Lethal of scute. (A) In wild-type embryos, Lethal of scute (L'sc, black) is expressed in about six midline cells (arrows) in each segment. (B) In wingless mutants, the number of Lethal of scute cells in a cluster (arrows) increases, up to 10 cells per segment. By contrast, in hedgehog mutants, Lethal of scute expression is lost (see Fig. 3B). (C) Cells taken from donors that express Wingless ubiquitously (V2H-GAL4/+; UAS-wg+/+) were transplanted next to the midline of wild-type embryos. Wingless-expressing cells (asterisk) repress Lethal of scute (dark purple) in the segment into which they integrate (arrowhead), whereas the expression of Lethal of scute in adjacent segments is not affected (arrows). The expression of Gooseberry-Distal (Gsb-d, brown), which serves as a segmental marker, does not change. All embryos are at stage 10. Ventral views, anterior to the left; brackets outline the midline.
Lethal of scute (data not shown, n=10). Hence, in contrast to Hedgehog, which activates Lethal of scute in midline cells, Wingless signalling represses Lethal of scute.

**Wingless maintains early Engrailed expression at the midline**

In *hedgehog* mutants and *lethal of scute* mutants, the early expression of Engrailed that is maintained from the blastoderm stage is never affected. We investigated whether this early expression of Engrailed depends on Wingless. At stage 9 in wild-type embryos, two to three midline cells are Engrailed positive (Fig. 5A). In *wingless* mutants, the expression of Engrailed after the division of midline precursors is not maintained, resulting in a complete absence of midline Engrailed at stage 9 (Fig. 5B). In *hedgehog* mutants, the normal number of midline cells express Engrailed, albeit at a slightly lower level than in wild type (Fig. 5C). In *wingless* mutants, the late phase of Engrailed is initiated at the correct time (stage 10), and shortly after germband retraction there are still three to five Engrailed-positive midline cells (Fig. 5E), about half of the number present in wild type (Fig. 5D). *hedgehog* mutants fail to initiate late Engrailed expression and, after germband retraction, only about two midline cells are still Engrailed positive (Fig. 5F).

Because Wingless counteracts Hedgehog signalling at the ventral midline by repressing Lethal of scute, we examined whether midline targeted expression of Hedgehog could increase the number of late Engrailed-expressing midline cells in *wingless* mutants. At stage 16, wild-type embryos have six to nine Engrailed midline cells per segment (Fig. 5G), whereas in *wingless* mutants only one to three Engrailed-positive midline cells per segment persist (Fig. 5H). Midline expression of Hedgehog in *wingless* mutants only slightly increases the number of Engrailed-positive cells (Fig. 5I). Ectopic expression of Hedgehog results in cell death, as indicated by the increased number of cell fragments (Fig. 5I), and the surviving midline cells are not integrated into the CNS.

Finally, we expressed tau-GFP in *wingless* mutants to analyse midline cell differentiation. In wild-type embryos, the differentiation of midline cells is far advanced at stage 14 (Fig. 5J). In *wingless* mutants, we detect no morphological differentiation and all midline cells are positioned at the dorsal CNS surface (Fig. 5K). After stage 14, cell death reduces the number of midline cells to a number comparable to that in *hedgehog* mutants (data not shown). We conclude that Wingless is essential to maintain early Engrailed expression after the division of midline precursors, whereas Hedgehog activates and induces late Engrailed at the ventral midline. In *wingless* mutants, as in *hedgehog* mutants, midline cells do not differentiate, and they die during late embryogenesis.

**Ectopic Hedgehog induces Lethal of scute and Engrailed in all midline cells and interferes with midline cell differentiation**

Because Hedgehog is needed to activate and maintain the expression of Lethal of scute and late Engrailed, we investigated whether ectopic expression of Hedgehog in the neuroectoderm and developing CNS (*sca-GAL4*) is sufficient to expand the expression

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**Fig. 5. Wingless and Hedgehog control different phases of Engrailed expression at the ventral midline.** (A) In wild-type, at stage 9, two to four midline cells per segment express Engrailed (En, dark purple). (B) In *wingless* (wg) mutants, Engrailed expression in the ectoderm and at the ventral midline is lost. (C) In *hedgehog* (hh) mutants, Engrailed expression in the midline weakens but persists. (D) At stage 13, Engrailed-positive midline cells form a prominent cluster. (E) In *wingless* mutants, despite the loss of early Engrailed expression, the late phase of Engrailed expression in the midline is initiated. (F) In *hedgehog* mutants, the late phase of Engrailed expression is never induced. The number of Engrailed-positive midline cells does not increase after stage 9. (G) In wild-type, Engrailed continues to be expressed in the midline until stage 17. (H) At stage 16, *wingless* mutants show a severe reduction in Engrailed-expressing midline cells. Most midline cells die. Surviving midline cells do not integrate into the CNS, resulting in the separation of the two sides of the CNS. (I) Ectopic expression of Hedgehog (Hh, brown) in all midline cells in *wingless* mutants only partially rescues the loss of Engrailed (black). Midline cells are excluded from the CNS. Cell fragments can be found in macrophages (arrows). (J) At stage 14, the expression of tau-GFP (GFP, brown) in all midline cells reveals an elaborate axon pattern. (K) In *wingless* mutants, midline cells are not integrated into the CNS and show no differentiation. Ventral views, anterior to the left; brackets outline the midline.
of these two genes. At the onset of stage 11, ectopic expression of Hedgehog induces Lethal of scute in all midline cells (compare Fig. 6A with 6B). At the end of stage 11, all midline cells also start to express Engrailed (compare Fig. 6C with 6D). For both Lethal of scute and Engrailed, the expression in the endogenous cluster is stronger than in cells in which ectopic expression is induced by Hedgehog (Fig. 6B,D). This is most likely due to continued repression by Wingless.

Midline cells are essential for the formation of commissures – axon bundles crossing the midline (reviewed by Jacobs, 2000). The first axons to cross the midline can be detected in the anterior commissure by staining for Futsch, the *Drosophila* orthologue of the vertebrate microtubule associated protein 1B (MAP1B; Fig. 6C). The Futsch-positive commissural axons are absent in embryos in which ectopic Hedgehog activates Engrailed in all midline cells (Fig. 6D). Also, the staining of all axons with the monoclonal antibody BP102 reveals that Hedgehog-induced Engrailed expression abolishes the formation of the anterior commissure, but not of the posterior commissure (compare Fig. 6E with 6F). Ectopic Hedgehog in the neuroectoderm and CNS also interferes with the differentiation of midline glia (compare Fig. 6G with 6H) and MP1 interneurons (compare Fig. 6I with 6J), midline subsets that in wild-type embryos do not express Engrailed.

We examined whether induction of Engrailed by ectopic Hedgehog is able to transform non-Engrailed-expressing midline subsets (midline glia, MP1 interneurons, UMI) into Engrailed-expressing midline subsets (VUM, MNB). In wild-type embryos, the expression of tau-GFP in midline cells outlines neurons and glial cells (Fig. 6K). Wingless expression in all midline cells from late stage 9 does not interfere with differentiation (Fig. 6L). Hedgehog expression in all midline cells activates Engrailed in all cells (Fig. 6M), and results in a severe reduction in cell number (Fig. 6N). The few remaining axons of the surviving midline cells suggest that the UMI and VUM interneurons are still able to differentiate. In conclusion, ectopic Hedgehog induces Lethal of scute and late Engrailed in all midline cells. Ectopic Hedgehog interferes with the

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**Fig. 6.** Ectopic expression of Hedgehog interferes with midline cell differentiation. (A) At stage 11, about six midline cells per segment express the proneural gene *lethal of scute* (L’sc, black). (B) Ectopic expression of Hedgehog in the neuroectoderm and developing CNS (sca-GAL4) induces Lethal of scute expression in all midline cells. (C) At stage 13, the first axons cross through the anterior commissure (arrow; Futsch, brown). Engrailed-positive midline cells (En, dark purple) cluster at the developing posterior commissure. (D) Ectopic expression of Hedgehog activates Engrailed expression in all midline cells and prevents the crossing of axons through the anterior commissure. (E) Axons (BP102, brown) in the mature CNS form a ladder with the anterior (arrowhead) and posterior commissure as rungs. (F) Ectopic expression of Hedgehog deletes the anterior commissure (arrowhead). (G) At the end of embryogenesis, midline glia cells (star; Slit, dark purple) tightly enwrap the commissures. (H) In embryos expressing Hedgehog ectopically, midline glia cells do not enwrap the remaining commissure. Often midline glia cells become apoptotic (arrow). (I) In each segment Odd (brown) is expressed in two midline-derived MP1 neurons and two dMP2 neurons. (J) Ectopic expression of Hedgehog eliminates Odd expression from most MP1 neurons (brown, arrow) but does not affect expression in the dMP2 neurons. (K) At the end of embryogenesis, midline cells (GFP, green) show an intricate axonal pattern and a subset of cells continues to express Engrailed (En, red). (L) The ectopic expression of Wingless in all midline cells does not interfere with the differentiation of the cells or the expression of Engrailed. (M) Ectopic expression of Hedgehog in all midline cells activates the ectopic expression of Engrailed in the midline. (N) Ectopic Hedgehog causes a severe reduction in the number of midline cells. Ventral views, anterior to the left; brackets outline the midline.
differentiation of most midline subsets but cannot transform non-Engrailed-expressing midline subsets into Engrailed-expressing subsets.

**Ectopic Engrailed expression in midline cells prevents the differentiation of midline glia and MP1 interneurons**

Hedgehog acts as a morphogen: different concentrations of Hedgehog can instruct different cell fates (reviewed by Hooper and Scott, 2005). Ectopic expression of Hedgehog in the neuroectoderm and the developing CNS, or in all midline cells, equalises the morphogen gradient and not only exposes midline cells to ectopic Hedgehog but also to concentrations that may be too high to allow their differentiation.

We created single sources of ectopic Hedgehog by transplanting Hedgehog-expressing cells (from V2H-GAL4/UAS-hh embryos) into embryos in which all midline cells are outlined by the expression of tau-β-galactosidase (Callahan and Thomas, 1994; Hidalgo et al., 1995). Transplantation of Hedgehog-expressing cells activates Engrailed expression in midline cells (n=6, Fig. 7A). The majority of transplanted cells (n=14) do not serve as an ectopic source of Hedgehog because they integrate into the endogenous Hedgehog domain. These cells fail to induce Engrailed. Cells transplanted as a control between wild-type embryos never induce Engrailed (n=8, data not shown).

Ectopic Hedgehog interferes with the differentiation of the MP1 interneurons (11/17 embryos). MP1 axons are missing (n=8), causing a gap in the MP1 axon fascicle (Fig. 7B), or the axons project randomly across the longitudinal tracts (n=3). The number of midline cells in the affected neuromeres is not reduced, although the midline glia are occasionally absent or pushed out of the CNS (2/17; data not shown). The axons of the VUM interneurons defasciculate slightly (2/17; data not shown). All other midline cell lineages develop normally. Transplantation of wild-type cells does not interfere with midline cell differentiation (n=5, data not shown).

We investigated whether the ectopic expression of Engrailed in midline cells is sufficient to explain the changes in midline cell differentiation caused by ectopic Hedgehog. Midline targeted expression of Engrailed from stage 10 mimics the phenotypes caused by the ectopic expression of Hedgehog in the neuroectoderm or in all midline cells. Ectopic Engrailed in midline cells prevents the formation of the anterior commissure (Fig. 7C,D), interferes with the differentiation of midline glia (Fig. 7E,F) and abolishes Odd expression in MP1 interneurons (Fig. 7G,H).

Finally, we labelled single midline precursors in embryos expressing Engrailed in all midline cells. Most unusually, nearly half of the labelled precursors (15/32) either generated only two undifferentiated cells (n=4) or the progeny died during embryogenesis (n=11). All the surviving progeny of the labelled precursors are abnormally positioned at the dorsal surface of the CNS. Midline cells expressing ectopic Engrailed rarely develop into midline glia (2/32 precursors) and the glial cells fail to enwrap the remaining, posterior, commissure (compare Fig. 8A with 8F). We never obtained MP1 interneurons (Fig. 8B), UMI neurons (compare Fig. 8C with 8G) showed only slight axonal pathfinding defects (3/32), and axons of VUM motoneurons were more severely affected than interneuronal axons (8/32; Fig. 8D,H,I). In wild-type embryos, VUM motoneuron axons bifurcate in the anterior commissure and the branches extend to both sides of the embryo (Fig. 8D). Ectopic Engrailed at the midline prevents the formation of the anterior commissure, and VUM motoneuron axons now turn randomly to one side of the CNS, where the axons bifurcate (Fig. 8H,I). Normally VUM interneuronal axons bifurcate in the posterior commissure (Fig. 8D). In spite of the presence of a posterior commissure, most of the VUM interneurons (5/8) also project to only one side (Fig. 8H). MNB progeny (4/32) show severely retarded axonal growth (Fig. 8E,J,K). The frequency with which the different subsets of neurons and glial cells are found in our clonal analysis suggests that the non-Engrailed-expressing subsets have not taken on the identity of the Engrailed-expressing subsets. Instead, ectopic expression of Engrailed in midline cells prevents the differentiation of midline glia and MP1 interneurons, and results in increased cell death. It is possible that the observed axonal defects in the other lineages are not cell autonomous but result from the loss of the anterior commissure or the loss of anterior midline subsets.
DISCUSSION

The ventral midline provides an ideal system for a comprehensive genomic approach to the development of a CNS organiser. This is because of the limited number of midline precursors, which give rise to only a few neuronal and glial progeny, and because of our complete knowledge of each of the lineages. Here, we show that the separation of midline cells into two compartments is an early and crucial step in midline cell determination. During germband elongation, a second phase of Engrailed expression is initiated at the midline in the anterior cells of the next posterior segment. During germband retraction, these cells join the anterior segment where they develop into posterior midline cells. Expression of late Engrailed depends on Hedgehog signalling and the proneural gene lethal of scute. Lethal of scute precedes Engrailed expression and is also activated by Hedgehog. Hedgehog and Wingless signalling counteract each other to define the position of the Lethal of scute cluster, and to divide the 16 midline daughter cells into eight non-Engrailed- and eight Engrailed-expressing cells.

Posterior midline cells are determined after the division of the precursors

It has generally been believed that the determination of the different subsets of midline cells occurs before the precursors undergo their simultaneous division at stage 8 (reviewed by Jacobs, 2000). This view is challenged by our observation that expression of the proneural gene lethal of scute, and the subsequent expression of Engrailed, is initiated in midline daughter cells at stage 10, about one hour after the precursors divide. In the neuroectoderm, proneural genes confer neural competence to a cluster of ectodermal cells (reviewed by Skeath and Thor, 2003). Lateral inhibition by Notch/Delta signalling then limits the expression of proneural genes to a single cell, which delaminates from the ectoderm and becomes a neural precursor (neuroblast). Because we have shown that the only neuroblast at the ventral midline (median neuroblast, MNB) originates from the proneural Lethal of scute cluster, it seems likely that the MNB is selected by lateral inhibition from a cluster of midline daughter cells. However, the process of lateral inhibition in the midline differs from that in the adjacent neuroectoderm. In the neuroectoderm, a single cell delaminates and the remaining cells of the cluster cease proneural expression and give rise to the epidermis (reviewed by Skeath and Thor, 2003). The proneural cluster in the midline consists of three pairs of siblings generated by the division of three separate precursors. Labelling of single precursors shows that, during the selection of the MNB, only one of the two, labelled siblings enlarges but both delaminate from the embryo (data not shown). In contrast to the neuroectoderm, the remaining cells of the midline cluster continue to express Lethal of scute after delamination of the MNB. This extended proneural expression might be necessary to maintain neural competence in the non-delaminating cells that develop into VUM neurons.

Fig. 8. Ectopic Engrailed expression in all midline cells affects the differentiation of all midline lineages. Midline precursors in wild type (A-E) and embryos expressing Engrailed in all midline cells (sim-GAL4/ UAS-En, F-K) were labelled with the fluorescent dye, Dil. After differentiation of the progeny, Dil was photoconverted to generate a permanent brown stain. The subset of lineages are indicated: MP1, midline precursor 1 neuron; UMI, unpaired median interneurons; VUM, ventral unpaired median neurons; MNB, median neuroblast neurons. Clones in F,J are also stained with anti-Engrailed (En, black). (A) Midline glia tightly enwrap the commissures. (E) Midline glia ectopically expressing Engrailed are found on the dorsal surface of the CNS. (B) The bifurcated axons of MP1 neurons project along the longitudinal tracts. We never obtained a clone resembling MP1 neurons in embryos expressing Engrailed in all midline cells. (C) The projection of one UMI (arrow) differs from the second UMI axon (arrowhead). (G) Expression of ectopic Engrailed in UMI only slightly affects their projections. (D) The axons of VUM motoneurons (arrow) bifurcate in the anterior commissure, whereas the VUM interneuronal axons (arrowhead) bifurcate in the posterior commissure. The cell bodies of VUM neurons are out of focus, at the ventral surface of the CNS. (H) When Engrailed is expressed ectopically in midline cells, all of the axons of the VUM motoneurons (arrow) exit the CNS on the same side. (I) Interneuronal axons (arrowhead) show the same phenotype. (E) The axons of MNB motoneurons (arrows) and interneurons (arrowhead) bifurcate in the anterior commissure. (J,K) Ectopic Engrailed in MNB neurons prevents or delays the bifurcation of their axons. Ectopic Engrailed expression on the midline causes all subsets to be mispositioned along the dorsal surface of the CNS. Ventral views, anterior to the left.
Our results cannot exclude the possibility that some of the midline subsets are determined as precursors, but we show that at least two of the five midline subsets, the VUM neurons and the MNB, are determined after precursor cell division. There are striking similarities between the development of the ventral midline of Drosophila and grasshopper embryos (Bastiani et al., 1985; Bossing and Technau, 1994). In grasshopper, Engrailed expression can be detected in the MNB, its progeny and the midline precursors MP4 to MP6, which each give rise to two neurons with projection patterns comparable to the Drosophila VUM neurons (Jia and Siegler, 2002). Hence, the same types of midline cells express Engrailed in grasshopper and Drosophila, but in grasshopper Engrailed expression is initiated in all midline precursors prior to division (Jia and Siegler, 2002).

The role of Hedgehog and Wingless in midline cell determination

In the ectoderm from stage 10 onwards, Wingless, Engrailed and Hedgehog maintain the expression of one another by a feedback loop: Wingless maintains Engrailed expression, Engrailed is needed for the expression of Hedgehog and Hedgehog maintains Wingless expression (Bejsovec and Martinez Arias, 1991; Ingham and Hidalgo, 1993). In the developing CNS, Wingless and Hedgehog expression seem to be independent of each other (Bhat, 1999). At the ventral midline there are two separate stages of Engrailed expression (Fig. 9): the early phase is maintained by Wingless (Fig. 9A); the late phase does not require Wingless and is instead activated at stage 10 by Hedgehog signalling and Lethal of scute (Fig. 9B,C). In the ectoderm, Wingless and Hedgehog act in concert to maintain Engrailed expression (Bejsovec and Martinez Arias, 1991), but at the midline Wingless and Hedgehog act in opposition: Wingless represses and Hedgehog activates Lethal of scute expression (Fig. 9B).

Wingless may repress Lethal of scute expression indirectly, via its maintenance of early Engrailed. As in the ectoderm, midline Engrailed represses expression of the Hedgehog receptor Patched and the Hedgehog signal transducer Cubitus interruptus (reviewed by St Johnston and Nusslein-Volhard, 1992). It is possible that early Engrailed-expressing midline cells are not able to receive the Hedgehog signal. However, ectopic expression of Hedgehog is able to induce Lethal of scute in all midline cells, suggesting that Wingless may repress Lethal of scute by a yet unknown mechanism. Recently it has been reported that a vertebrate wingless orthologue, Wnt2b, can maintain the naïve state of retinal progenitors by attenuating the expression of proneural and neurogenic genes (Kubo et al., 2005).

We examined the differentiation of midline cells in wingless and hedgehog mutants. Consistent with earlier reports (Hummel et al., 1999), many midline cells become apoptotic in both mutants. The surviving midline cells are not integrated into the CNS and show no morphological differentiation. The reduction in the number of Engrailed-positive midline cells in hedgehog mutant embryos may be mainly due to the loss of midline cell identity. In hedgehog mutants, midline cells lose the expression of Sim, the master regulator of midline development (reviewed by Crews, 1998). As described for sim mutants, the loss of midline identity results in increased cell death and mis-specification of the surviving midline cells as ectoderm (Xiao et al., 1996).

Ectopic Hedgehog induces the expression of Lethal of scute and late Engrailed in all midline cells

Ectopic expression of Hedgehog in the neuroectoderm and the developing CNS induces the expression of Lethal of scute and, approximately 40 minutes later, the expression of late Engrailed in all midline cells. It seems likely that Lethal of scute is an early target of Hedgehog signalling, and its activation may only require release from repression by the short form of Cubitus interruptus (Methot and Basler, 1999; Muller and Basler, 2000). By contrast, the delay in induction of late Engrailed in the same midline cells indicates that Engrailed activation may not only require release from repression, but also activation by the long form of Cubitus interruptus (reviewed by Hooper and Scott, 2005).

Uniformly high levels of ectopic Hedgehog prevent the differentiation of most midline subsets and cause increased cell death. A single source of ectopic Hedgehog, achieved by cell transplantation, does not result in midline cell death, but reveals that the differentiation of the MP1 interneurons is more sensitive to Hedgehog levels than is the differentiation of midline glia. No other midline subsets are affected. It seems likely that Hedgehog not only activates Lethal of scute and late Engrailed, but also acts as a morphogen to control the differentiation of the MP1 neurons and midline glia.

**Fig. 9.** Opposing functions of Hedgehog and Wingless signalling separate midline siblings into an anterior and posterior compartment. **(A)** At stage 9, after the division of the midline precursors, Wingless signalling maintains Engrailed expression in two midline siblings. **(B)** At stage 10, repression by Wingless and activation by Hedgehog positions the proneural Lethal of scute cluster directly posterior to the early Engrailed-expressing cells. **(C)** Expression of Hedgehog and Lethal of scute are needed to induce and maintain late Engrailed expression in the midline siblings. **(D)** The induction of Engrailed by Hedgehog divides midline cells into two compartments. The absence of Engrailed in the anterior compartment allows Notch/Delta signalling to select between the MP1 interneuron and midline glial fate; Engrailed expression in the posterior compartment restricts the selection of cell fate by Notch/Delta signalling to MNB and VUM neurons. Midline cells at the parasegmental border, in the middle of the segment, may be determined as precursors.
Engrailed prevents the differentiation of midline glia and MP1 interneurons

The phenotypes caused by ectopic Hedgehog are due to the induction of Engrailed in all midline cells. Expression of ectopic Hedgehog and ectopic Engrailed blocks the differentiation of midline glia and MP1 interneurons, and also prevents the formation of the anterior commissure. Labelling single midline precursors enabled us to examine cell fates in embryos expressing ectopic Engrailed in the midline. The frequency of clones obtained indicates that ectopic Engrailed expression does not transform non-Engrailed-expressing midline subsets (MP1 interneurons, midline glia and UMI) into Engrailed-expressing subsets (VUM and MNB). Instead, embryos expressing midline Engrailed show increased cell death. In particular, the MP1 interneurons seem to be affected and were never obtained during our analysis. The low frequency of midline glia also points to apoptosis caused by expression of Engrailed. Surviving midline glia are not able to differentiate properly and cannot enwrap the remaining, posterior, commissure. All other midline subsets, including the UMI, are able to differentiate, but they show a variety of axonal pathfinding defects that may result from the loss of anterior midline subsets and the absence of the anterior commissure.

A model for midline cell determination

It is likely that genes other than hedgehog and wingless are crucial for midline cell determination. In our experiments, non-Engrailed-expressing midline subsets are never transformed into Engrailed-expressing subsets, or vice versa. gooseberry-distal may be one of these genes. From the blastoderm stage, Gooseberry-distal is expressed by two midline precursors and their four daughter cells. During early embryogenesis Gooseberry-distal expression at the midline does not depend on Wingless and Hedgehog (Bhat and Schedl, 1997). The anterior Gooseberry-distal cells also express Wingless and most likely give rise to the UMI. The posterior Gooseberry-distal pair also express early Engrailed and Hedgehog, and develop into the most posterior VUM neurons. At stage 10, Hedgehog activates the expression of Lethal of scute and Engrailed in midline cells posterior to the Gooseberry-distal domain. Lateral inhibition by Notch/Delta signalling selects one cell from the Lethal of scute cluster to become the MNB. The remaining cells become VUM neurons. At stage 10, the absence of Engrailed in the six midline cells anterior to the Gooseberry-distal domain defines a cell cluster that will give rise to midline glia and MP1 interneurons. Based on the expression of Odd, Delta mutants have an increased number of MP1 interneurons, up to six per segment (Spana and Doe, 1996). In Notch mutants, midline glial-specific markers are absent and the number of cells expressing a neuronal marker increases (Menne and Klambt, 1994). Therefore, Notch/Delta signalling appears to determine midline glial versus MP1 interneuron cell fates in the anterior cluster. In our model, midline cell determination takes place mainly after the division of the precursors. Although the initial determination of midline cells appears to be directed by a small number of genes, a far larger number is needed to control the differentiation of the various midline subsets. Our work, and the recent identification of more than 200 genes expressed in midline cells (Kearney et al., 2004), is the beginning of a comprehensive understanding of the differentiation of the ventral midline.

References


