

The CNS midline cells and *spitz* class genes are required for proper patterning of *Drosophila* ventral neuroectoderm

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ABSTRACT The *Drosophila* embryonic central nervous system (CNS) develops from sets of neuroblasts (NBs) which segregate from the ventral neuroectoderm during early embryogenesis. It is not well established how each individual NB in the neuroectoderm acquires its characteristic identity along the dorsal-ventral axis. Since it is known that CNS midline cells and *spitz* class genes (*pointed*, *rhomboid*, *single-minded*, *spitz* and *Star*) are required for the proper patterning of ventral CNS and epidermis originated from the ventral neuroectoderm, this study was carried out to determine the functional roles of the CNS midline cells and *spitz* class genes in the fate determination of ventral NBs and formation of mature neurons and their axon pathways. Several molecular markers for the identified NBs, neurons, and axon pathways were employed to examine marker gene expression profile, cell lineage and axon pathway formation in the *spitz* class mutants. This analysis showed that the CNS midline cells specified by *single-minded* gene as well as *spitz* class genes are required for identity determination of a subset of ventral NBs and for formation of mature neurons and their axon pathways. This study suggests that the CNS midline cells and *spitz* class genes are necessary for proper patterning of the ventral neuroectoderm along the dorsal-ventral axis.

KEY WORDS: *Drosophila*, ventral neuroectoderm patterning, CNS midline cells, *spitz* class genes

Introduction

One of the most important goals in developmental neurobiology is to understand how cellular diversity of neurons and glia is generated and how their intricate cellular networks are formed. The fruit fly, *Drosophila melanogaster*, has been selected to study the molecular basis of neurogenesis because of its sophisticated genetics, a relatively simple central and peripheral nervous system (CNS and PNS), and the recent advent of molecular genetic and cellular technology.

The *Drosophila* CNS develops from the ventral neuroectoderm (VNE), also called the neurogenic region (Campos-Ortega and Hartenstein, 1997). The neurogenic region is bilaterally symmetrical, and is divided into left and right hemisegments by a narrow strip of specialized midline cells, the mesectodermal cells (Crews *et al.*, 1988; Thomas *et al.*, 1988). Neurogenesis begins with delamination of neuroblasts (NBs) and glioblasts (GBs) from the VNE, and NBs form a two-dimensional subepidermal array composed of approximately 30 NBs per hemisegment. The remaining cells of the neurogenic region become the ventral epidermis (VE). Each NB divides asymmetrically to generate smaller ganglion mother cells (GMCs), and then each GMC divides once to

produce a pair of neurons and/or glia (Cabrera, 1992; Goodman and Doe, 1993; Bossing *et al.*, 1996; Schmidt *et al.*, 1997). Individual NBs undergo a characteristic temporal and spatial delamination process in each segment. NB formation occurs in five pulses (S1-S5), beginning about 30 min after gastrulation (Fig. 1; Doe, 1992; Broadus *et al.*, 1995). The first 10 S1 NBs form an orthogonal array of four rows and three columns (medial, intermediate, and lateral) in each segment. Approximately within 30-40 min intervals, a new group of NBs delaminates: five S2 NBs, five S3 NBs and one GB, five S4 NBs and six S5 NBs, generating a total of 31 NBs and 1 GB per hemisegment. This stereotyped pattern of NB delamination suggests that each NB acquires its unique identity based on its location within the neuroectoderm.

Extensive studies of molecular mechanisms of neurogenesis have revealed that two classes of genes play important roles in NB

Abbreviations used in this paper: CNS, central nervous system, NB, neuroblast; PNS, peripheral nervous system; GB, glioblast; VNE, ventral neuroectoderm; VE, ventral epidermis; GMC, ganglion mother cells; A-P, anterior-posterior; D-V, dorsal-ventral; EGF, epidermal growth factor; MP2, median precursor 2; β -gal, β -galactosidase; VUM, ventral unpaired median; SN, segmental nerve; ISN, intersegmental nerve.

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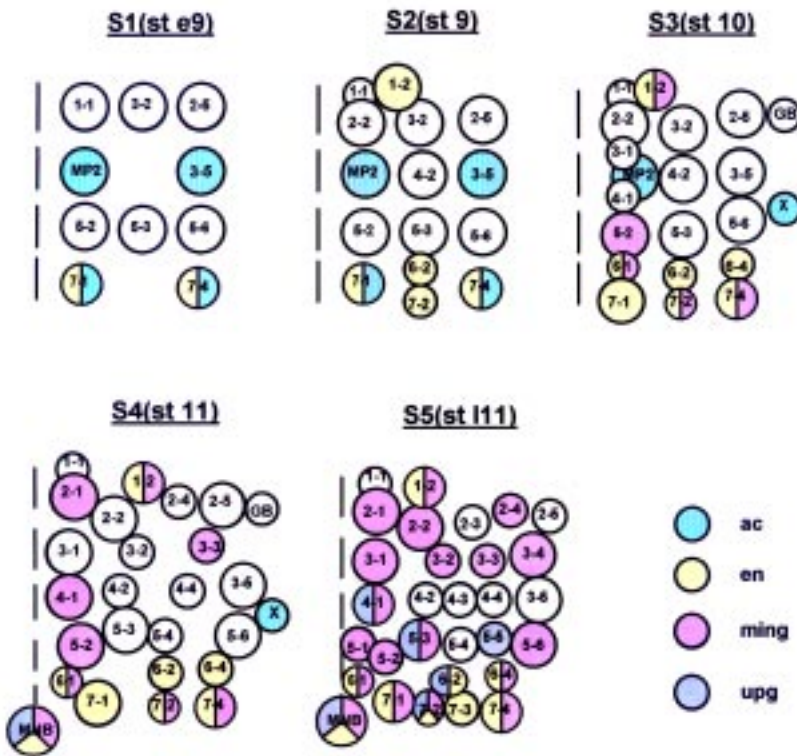


Fig. 1. Expression pattern of NB markers. Summary of the consensus pattern of NB at different developmental time points with the expression of four different molecular markers indicated in different colors. S1, early stage 9; S2, stage 9; S3, stage 10; S4, stage 11; S5, late stage 11. Stages are from Campos-Ortega and Hartenstein (1997). Markers: ac, achaete; en, engrailed; ming, ming; upg, unplugged. Heading, NB stage; top, anterior; dotted line, ventral midline (Adopted from Broadus et al., 1995).

formation: the proneural genes control the competence of a group of neuroectodermal cells to become NBs and the neurogenic genes control the cellular interactions that ensure only a single cell in the group gives rise to a NB (Campos-Ortega, 1993). Although the mechanisms for NB formation are relatively well understood, the molecular mechanisms leading to the determination of individual NB identity and its lineage along the anterior-posterior (A-P) and dorsal-ventral (D-V) axis are poorly established. Several lines of evidences have suggested that NBs are generated both by invariant positional cues within the neuroectoderm that depend on developmental history and cellular interactions between neuroectodermal cells along the A-P and D-V axis (Thomas et al., 1984; Cabrera, 1992; Goodman and Doe, 1993). It is known that a number of pair rule and segment polarity genes are involved in NB fate determination by assigning positional cues in the VNE along the A-P axis (reviewed by Bhat, 1998). The secreted Wingless protein was shown to be required non-autonomously for adjacent anterior and posterior NB fate determination and formation (Chu-LaGraff and Doe, 1993), and another segment polarity gene, *gooseberry-distal*, was demonstrated to be necessary and sufficient for the formation of row 5 NBs (Zhang et al., 1994; Skeath et al., 1995). It was recently shown that *hedgehog* and *patched* are involved in fate determination and formation of a subset of specific NBs (Bhat, 1996; Matsuzaki and Saigo, 1996; McDonald and Doe, 1997). Despite these advances in understanding the molecular genetic regulatory mechanisms of NB formation along the A-P axis, it is not well known how individual NBs

acquire their characteristic identities along the D-V axis. It was recently reported that the VNE is subdivided into three columns (medial, intermediate, lateral) along the D-V axis by the expression of *escargot* in both medial and lateral columns (Yagi and Hayashi, 1997), *ventral nervous system defective (vnd)/Nkx-2* (Jimenez et al., 1995; Mellerick and Nirenberg, 1995) and *muscle-specific homeodomain (msh)/Msx* (D'Alessio and Frasch, 1996; Wang et al., 1996; Isshiki et al., 1997) in the medial and lateral column, respectively. It was also shown that *Drosophila epidermal growth factor receptor (Egfr)* is responsible for the formation of intermediate NBs, specification of medial NBs, and repression of lateral NB fate in the medial and intermediate region of VNE, resulting in subdivision into three D-V domains of the proneural clusters during the gastrulation stage (Skeath, 1998; Udolph et al., 1998; Yagi et al., 1998).

The *spitz* class gene family is one of the best candidates for providing positional specification along the D-V axis in the VNE. This family includes *pointed (pnt)*, *rhomboid (rho)*, *single-minded (sim)*, *spitz (spi)* and *Star (S)*. They show common phenotypes such as the loss of VE and decrease in spacing between the longitudinal and commissural connectives (Mayer and Nüsslein-Volhard, 1988), suggesting that they affect fate determination of both CNS and VE that are generated from the VNE. It was demonstrated using a number of VE-specific molecular markers that *Egfr* and *orthodenticle (otd)* genes as well as the *spitz* class genes are also involved in VE-specific gene expression and differentiation (Kim and Crews, 1993; Raz and Shilo, 1993). It was recently shown that the CNS midline cells are required for the differentiation of lateral neurons and formation of specific mesodermal cells (Lüer et al., 1997; Menne et al., 1997; Zhou et al., 1997).

Molecular genetic characterization of the *spitz* class genes has provided useful information on the role of each gene in the establishment of NB and VE fate. The *sim* gene product is known to be a nuclear protein containing a basic helix-loop-helix motif and is essential for midline expression and formation of CNS midline cells (reviewed by Crews, 1998). A wide range of genetic interactions occurring during oogenesis, wing vein, and eye photoreceptor formation strongly support the idea that *Egfr*, *pnt*, *rho*, *spi*, and *S* act together as an EGF/TGF- α signaling cassette in a common pathway of several developmental processes (reviewed by Freeman, 1997; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). *spi* encodes a homolog of the vertebrate EGF receptor ligand, transforming growth factor- α (TGF- α) (Rutledge et al., 1992), and the secreted form of Spi activates the EGF receptor signaling pathway (Schweitzer et al., 1995; Gabay et al., 1997). It was proposed that the CNS midline cells are the site for processing of Spi required for proper ventral ectodermal patterning (Golembo et al., 1996). The Rho and S proteins contain seven transmembrane domains and a single transmembrane domain, respectively (Bier et al., 1990; Kolodkin et al., 1994). Considering the restricted expression pattern of *rho* and wide expression pattern of *spi* and *Egfr* in the neuroectoderm (Bier et al., 1990; Zak et al., 1990; Rutledge et al., 1992), it was proposed that the restricted expression pattern of *rho* may provide spatial cues by

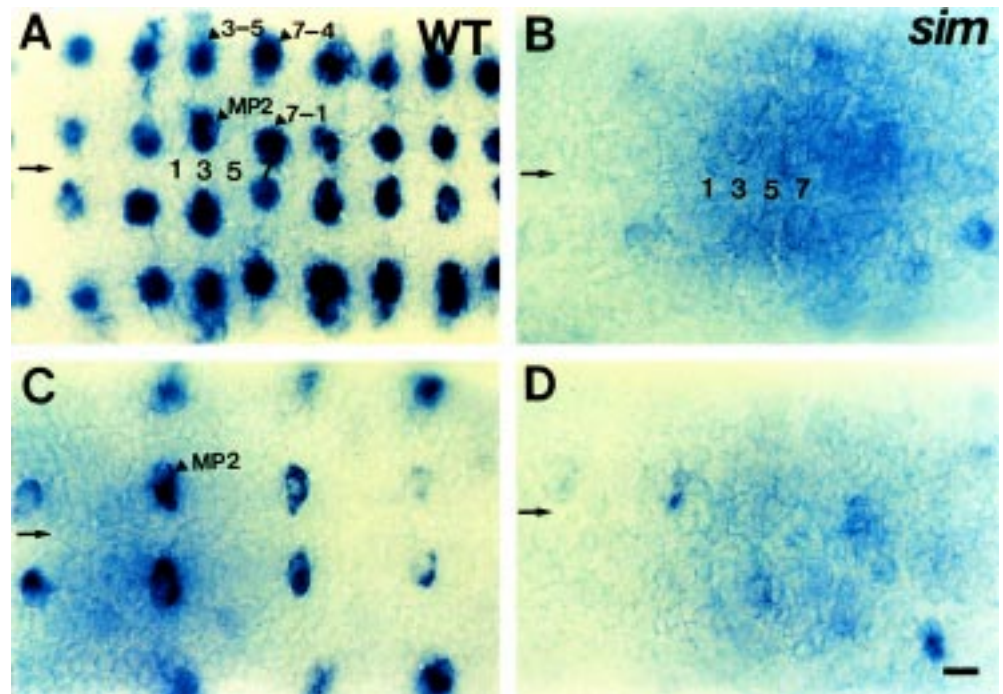


Fig. 2. The CNS midline cells are required for the proneural gene *ac* expression. (A,C) Wild-type *ac* expression in the MP2 and SI NBs 3-5, 7-1, and 7-4. **(A)** In a stage 9 wild-type embryo, MP2 and NB 7-1 are located in the medial 3rd and 7th rows per segment, respectively, and MP2 is slightly bigger than the other NBs. **(C)** In a stage 10 wild-type embryo, *ac* is expressed in MP2 and lateral NB with unknown origin in each segment. **(B,D)** *ac* expression in *sim* mutant embryos. *ac* expression is barely detectable in a stage 9 **(B)** and in a stage 10 *sim* embryo **(D)**. All panels show the ventral view of embryos with the ventral midline marked with an arrow, and anterior is to the left. In panels A and B, rows 1, 3, 5, and 7 are indicated. Bar, 22.5 μ m.

modulating the processing of the Spi ligand in many developmental processes (Sturtevant *et al.*, 1993). *pnt* encodes two related transcription factors with Ets domains and they were shown to be downstream targets of the EGF receptor signaling pathway in the formation of eye photoreceptor, ventral ectoderm and oocytes (Klämbt, 1993; Brunner *et al.*, 1994; Gabay *et al.*, 1996; Morimoto *et al.*, 1996). The *otd* gene product is a homeodomain-containing protein and is known to be required for medial VE cell fate determination (Finkelstein *et al.*, 1990; Wieschaus *et al.*, 1992). In summary, the molecular structures and genetic interactions of the *spitz* class genes in pathways encompassing a wide variety of developmental processes strongly suggest that they play important roles in ventral NB fate determination by cell-cell interactions via an EGF receptor signaling pathway. Considering the parallel defects in both CNS and VE and the potential roles of the *spitz* class genes in cell signaling, it was decided to investigate how the CNS midline cells affect patterning of the VNE.

In this study, several NB and neuronal molecular markers have been employed to examine the roles of the CNS midline cells and *spitz* class genes in patterning of the VNE along the D-V axis. It was shown here that the CNS midline cells specified by the *sim* gene are required for the fate determination of ventral NBs, formation of neurons, and establishment of proper axon pathways. The other *spitz* class genes also play important roles in this process. These results suggest that the CNS midline cells and *spitz* class genes are involved in proper patterning of the VNE along the D-V axis.

Results

The CNS midline cells are necessary for the proneural gene expression

NB formation and fate determination depend on the function of the *achaete-scute* complex of proneural genes, *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*) to provide a group of neuroectodermal cells with competence to become a NB at the specific

position and time (Campos-Ortega, 1993; Parras *et al.*, 1996; Skeath and Doe, 1996). These genes are initially expressed within the neuroectoderm in partially overlapping arrays of proneural clusters of about six cells. One NB develops from each cluster and the remaining ectodermal cells of the cluster lose gene expression during the S1-S2 stages of neurogenesis (Romani *et al.*, 1989; Martin-Bermudo *et al.*, 1991; Skeath and Carroll, 1992). To investigate whether the CNS midline cells affect the proneural gene expression essential for NB formation and fate determination, expression pattern of *ac* gene product, T5 RNA, was analyzed by *in situ* hybridization. In wild-type embryos, *ac* is expressed in the MP2 and NBs 3-5, 7-1, and 7-4 per hemisegment at stage 9 (Fig. 2A), and then in MP2 at stage 10 (Fig. 2C). In *sim* embryos, *ac* expression is absent in 95% of hemisegments (the number of hemisegments examined, $n=122$), although residual *ac* expression is occasionally observed (Fig. 2B,D). This result indicates that the CNS midline cells are required for proneural gene, *ac* expression in the medial and lateral NBs from the initial stage of NB formation. The absence of *ac* expression in the orthogonal array of NBs may reflect the change of NB identity or defect in NB formation and division.

The CNS midline cells are required for the expression of the S1-S5 NB markers that are essential for NB fate determination

To determine whether the absence of *ac* expression in the *sim* mutant is due to NB fate change or defects in NB formation and division, several markers for NB 7-1 were chosen to examine expression pattern in NB 7-1. *en* starts to be expressed in S1 NBs 7-1 and 7-4, and then in S2-S5 NBs 1-2, 6-1, 6-2, 6-4, 7-2, and 7-3 located in the 6th and 7th rows in each segment (Fig. 3A; Doe, 1992; Broadus *et al.*, 1995). At stage 13, *en* is expressed in three clusters of neurons in the posterior half of each segment: midline neuroblast cluster, bilateral clusters of 2-3 medial neurons and of 10-12 lateral neurons (Fig. 3C). At stage 16, *en* is expressed in the midline neuroblast cluster, bilateral clusters of 6 medial neurons

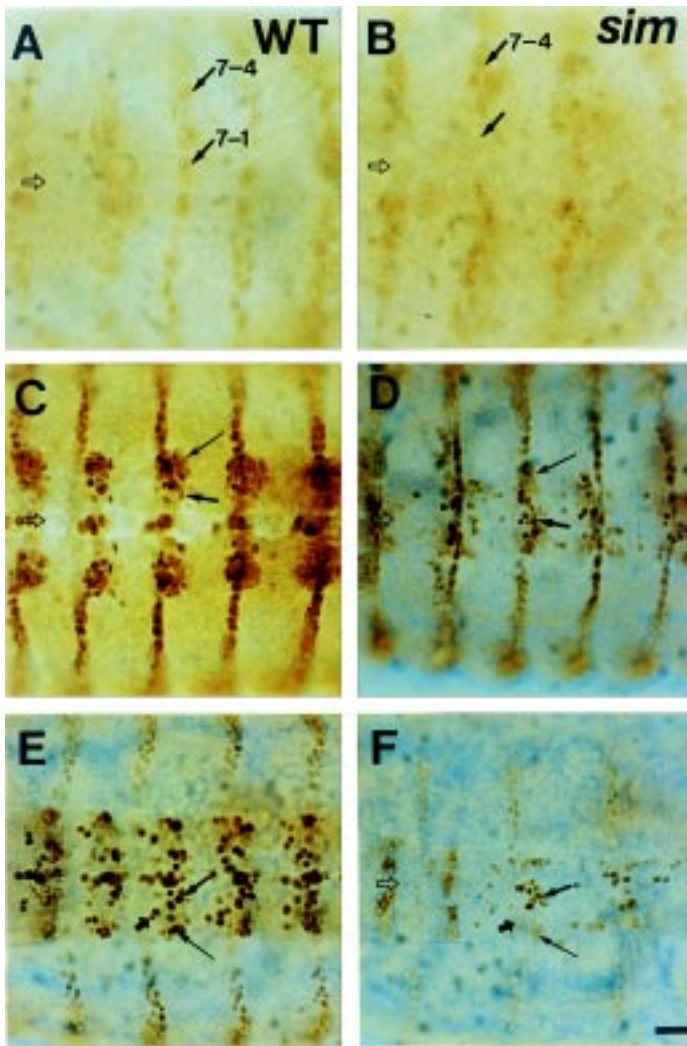


Fig. 3. The CNS midline cells are also required for *en* expression in S1 NBs 7-1 and 7-4, GMCs, and neurons during neurogenesis. (A,B) *en* expression at stage 9. *en* expression in S1 NB 7-1 (arrow) of a wild-type embryo (A) is missing in *sim* embryos (B). (C,D) *en* expression at stage 13. *en* expression in bilateral clusters of 10-12 lateral neurons (thin arrow) of a wild-type embryo (C) become reduced to 3-4 lateral neurons in *sim* embryos (D). On the other hand, *en* expression in the mediolateral neurons (short arrow) remain intact. (E,F) *en* expression at stage 15. (E) *en* expression of a wild-type embryo is shown in the bilateral clusters of 6 mediolateral (short arrow), 6 lateral (long arrow), and 2 anteroventral neurons (thick arrow) in each hemisegment as well as the midline neurons. (F) In the *sim* embryo, *en* expression is absent in the midline neurons and is shown in 2-3 lateral neurons per segment. All panels show ventral views with the ventral midline marked with an open arrow, and anterior is to the left. Bar, 22.5 μ m.

and of 6 lateral neurons (Fig. 3E). In addition, a pair of lateral neurons located at the ventral side of the ventral nerve cord show *en* expression. In stage 9 *sim* embryos, *en* expression in NB 7-1 is absent in 47% of hemisegments ($n=103$, Fig. 3B). Later in stage 13 and 16 *sim* embryos, the number of *en*-positive lateral neurons is reduced by half (Fig. 3D,F), and *en* expression in a bilateral pair of ventral neurons is reduced (Fig. 3F).

It was shown that *ming/lacZ* is a useful marker for NBs delaminating at the S3-S5 stages of neurogenesis (Cui and Doe,

1992; Broadus et al., 1995). *ming/lacZ* marker was used to determine whether the CNS midline cells affect marker gene expression in NB 7-1 and the other NBs delaminating at later S3-S5 stages after the initial round of neurogenesis has begun. *ming/lacZ* expression starts in a subset of the CNS midline cells at stage 9, and slightly later at early stage 10 appears in a single NB 6-1. By stage 10, *ming/lacZ* is expressed in five S3 NBs 1-2, 5-2, 6-1, 7-2, and 7-4, then in three additional S4 NBs 2-1, 3-3, and 4-1 at stage 11, and finally in a total of 18 S5 NBs (Fig. 4A). In stage 11 *sim* embryos, *ming/lacZ* expression is severely reduced especially in all the midline cells and 90% of medial NBs 2-1, 3-1, 4-1, and 5-1, intermediate NBs 1-2 and 2-2, and lateral NB 2-4 as shown in Figure 4B ($n=150$). On the other hand, *ming/lacZ* expression in NBs 5-2, 6-1, and 7-1 still remains. At stage 15, *ming/lacZ*-expressing neurons become fused at the ventral midline, resulting in a narrow ventral nerve cord (Fig. 4C,D). Another S5 NB marker, *upg/lacZ* expression begins in the midline NB at stage 11 and shows in S5 NBs 4-1, 5-3, 6-2, and 7-2 at late stage 11 (Chiang et al., 1995; Fig. 5A). In *sim* embryos, *upg/lacZ* expression is absent in 95% of S5 NB 4-1, and much reduced in the remaining NBs at late stage 11 ($n=128$; Fig. 5B) and in many neurons at stage 15 (Fig. 5D). *upg/lacZ*-expressing neurons appear to degenerate by stage 15 (Fig. 5C,D).

Taken together, these analyses showed that in *sim* mutant embryos NB 7-1 fails to express *ac* and *en* genes but still expresses *ming/lacZ*, indicating that the identity of NB 7-1 may have not been properly attained. These results suggest that the CNS midline cells are required for the expression of genes necessary for the proper identity determination of medial NBs during the S1-S5 stages of neurogenesis.

The CNS midline cells and *spitz* class genes affect the formation of GMCs and neurons during mid-stage neurogenesis

To determine whether the CNS midline cells influence the formation of the ventral GMCs and neurons derived from the NBs that underwent several rounds of cell division, *eve* expression patterns in many identified GMCs and neurons were analyzed by staining with the anti-Eve antibody. In wild-type embryos, *eve* is first expressed in five GMCs derived from at least three different NBs in each hemisegment at late stage 11; NB 1-1 gives rise to aCC and pCC neurons, NB 4-2 to RP2 and NB 7-1 to two CQ neurons (Fig. 6A, Broadus et al., 1995). Thus, examination of *eve* expression in the CQ neurons derived from S1 NB 7-1 would provide useful information on whether the loss of *ac* and *en* expressions in the NB 7-1 is due to NB formation or NB identity change. In stage 11 *sim* embryos (Fig. 6G), this analysis reveals the presence of *eve*-expressing CQ neurons derived from the NB 7-1 that shows no expressions of *ac* (Fig. 2A,B) and *en* (Fig. 3A,B) in stage 9 but shows weak *ming/lacZ* expression in stage 11 *sim* mutants (Fig. 4A,B). In addition, all the RP2 neurons do not express *eve*, but the other GMCs and neurons show normal *eve* expression. The aCC/pCC and CQ neurons become fused at the midline, and their cell number is similar to that of the wild-type embryos. Later in stage 15 *sim* embryos, *eve* expression in RP2 neurons remains in 45% of hemisegments ($n=192$). In the other *spitz* class mutants at stage 11, *eve* expression in RP2 neurons is reduced, and the number of *eve*-positive CQ neurons become reduced by half compared to that of the wild-type embryos especially in *Egfr*, *otd*, *pnt*, *S* and *spi* mutants (Fig. 6B-F,H). This

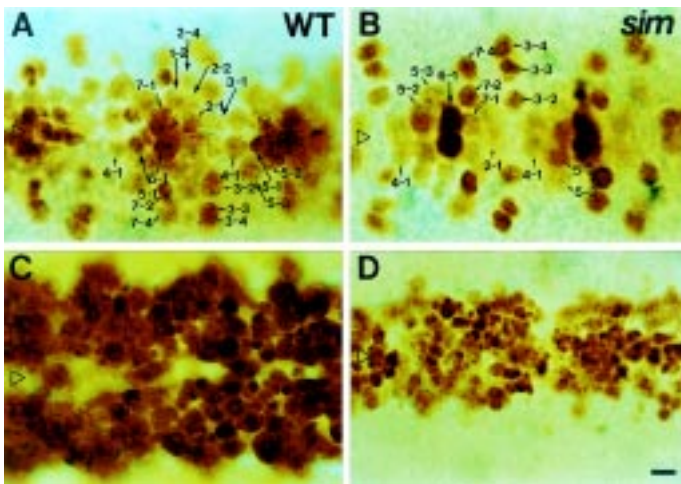


Fig. 4. The CNS midline cells are required for the expression of *ming/lacZ* S3-S5 NB marker. (A) *ming/lacZ* expression in a late stage 11 wild-type embryo showing a total of 15 S5 NBs including NBs 1-2, 2-1, 2-2, 2-4, 3-1, 3-2, 3-3, 3-4, 4-1, 5-1, 5-2, 5-3, 7-1, 7-2, and 7-4. (B) *ming/lacZ* expression in medial and intermediate NBs 1-2, 2-2, 2-4, 3-1, and 5-1 of a late stage 11 *sim* embryo is absent, although residual β -galactosidase expression remains in NBs 2-1, 4-1, and 7-1. (C) *ming/lacZ* expression in a stage 15 wild-type embryo is shown in dividing GMCs and neurons in the developing ventral nerve cord. (D) *ming/lacZ* expression in a stage 15 *sim* embryo shows the fused and narrow ventral nerve cord at the midline. All panels show the ventral view with the ventral midline marked with an open arrowhead, and anterior is to the left. Bar, 15.0 μ m.

result indicates that the correct number of CQ neurons are derived from NB 7-1, and they are still present in normal position, divide and differentiate in the *sim* mutant. This observation suggests that NB formation or division is not severely defective, but the identity of S1 NB 7-1 is altered in the *sim* mutant. It also shows that the CNS midline cells and *spitz* class genes also affect the formation of ventral GMCs and neurons.

To examine precisely the effects of the *sim* gene on the formation of aCC/pCC and RP2 neurons, the enhancer trap lines A31 and rG335 which label aCC/pCC and RP neurons, respectively, were employed. The A31 enhancer trap line has a P[*lacZ*] insert in the fasciclin II locus, and shows β -gal expression in the aCC and pCC neurons from stage 11 (Grenningloh *et al.*, 1991). The A31 enhancer trap line shows β -gal expression in the aCC and pCC per segment at stage 15 (Fig. 7A). In *sim* mutant embryos, the aCC and pCC neurons become fused at the midline but the number of neurons appears to remain almost the same as that of the wild-type (Fig. 7B), which is consistent with the previous result obtained with anti-Eve antibody (Fig. 6G). Another enhancer trap line rG335 was used to follow the RP neurons. The rG335 enhancer trap line shows stronger β -gal expression in the RP neurons and weaker expression in the RP2 neurons located medial to the midline cells (Fig. 7C) at stage 15. In stage 15 *sim* embryos, β -gal expression in the RP2 neurons disappears in more than 84% of hemisegments ($n=138$, Fig. 7D), confirming the previous result obtained by using anti-Eve antibody staining (Fig. 6G). This result also indicates that the identity of remaining 45% of *eve*-positive RP2 neurons at stage 15 is not correctly assigned in *sim* mutant embryos.

Taken together, these analyses demonstrated that the NB 7-1, which is devoid of *ac* and *en* expression but maintains *ming/lacZ*

expression in the *sim* mutant, is still present and gives rise to a normal number of CQ neurons. This result indicates that the loss of marker gene expressions in the NB 7-1 of *sim* embryos is largely due to NB identity change. It is also suggested that the CNS midline cells and the *spitz* class genes affect the identity determination of GMC 4-2 and RP2 neurons, as detected by the loss of marker gene expressions in the identified GMCs and neurons.

The *spitz* class genes are also required for expression of NB marker genes during the S3-S5 stages of neurogenesis

To demonstrate whether the other *spitz* class genes are involved in proper expression of the genes required for fate determination of NBs located lateral to the CNS midline cells, β -galactosidase expression patterns of *ming/lacZ* and *upg/lacZ* lines were examined in the *spitz* class mutants. The expression of *ming/lacZ* was clearly missing in many medial and intermediate NBs of the *spitz* class mutant embryos at stage 11 (Fig. 8A,C,E,G,I). *spt* and *S* mutant embryos show more severe reduction in *ming/lacZ*-expressing NBs than in *pnt* and *rho* mutants. *upg/lacZ* expression in the *spitz* class mutants is much reduced in NBs 4-1 and 5-3 at stage 12 (Fig. 8B,D,F,H,J). These results indicate that the *spitz* class genes are required for the expression of NB markers necessary for the identity determination of NBs that are located medial and intermediate to the CNS midline cells during the S3-S5 stages of neurogenesis.

The CNS midline cells and *spitz* class mutants affect axon pathway formation during late neurogenesis

Since the CNS phenotype of the *spitz* class mutants shows common characteristic features of fused anterior and posterior commissures and decreased spacing between the longitudinal connectives (Mayer and Nüsslein-Volhard, 1988), the axon pathways of the CNS were examined using the anti-Fas II antibody. The

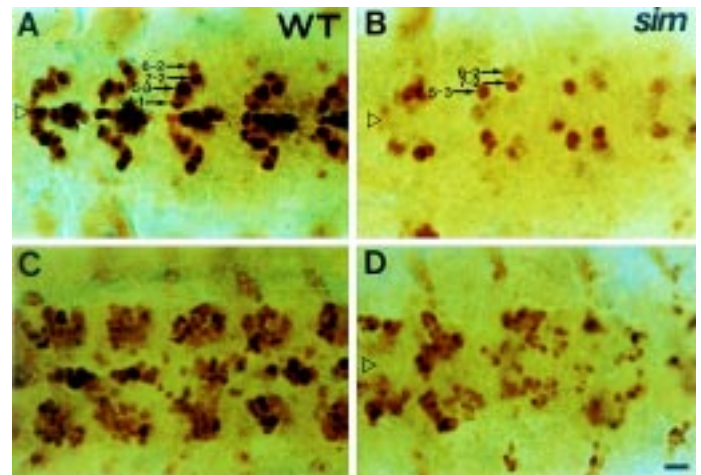


Fig. 5. The CNS midline cells are required for the expression of *upg/lacZ* S5 NB marker. (A) *upg/lacZ* expression in a late stage 11 wild-type embryo showing a total of 5 S5 NBs including 4-1, 5-3, 6-2, and 7-2 (arrows from the midline to lateral). (B) *upg/lacZ* expression is absent in the medial NB 4-1 and reduced in the other NBs in a late stage 11 *sim* embryo. (C) *upg/lacZ* expression in a stage 15 wild-type embryo representing clusters of lateral neurons. (D) *upg/lacZ* expression in the clusters of lateral neurons becomes absent and disorganized in a stage 15 *sim* embryo. All panels show ventral views with the ventral midline marked with an open arrowhead, and anterior is to the left. Bar, 15.0 μ m.

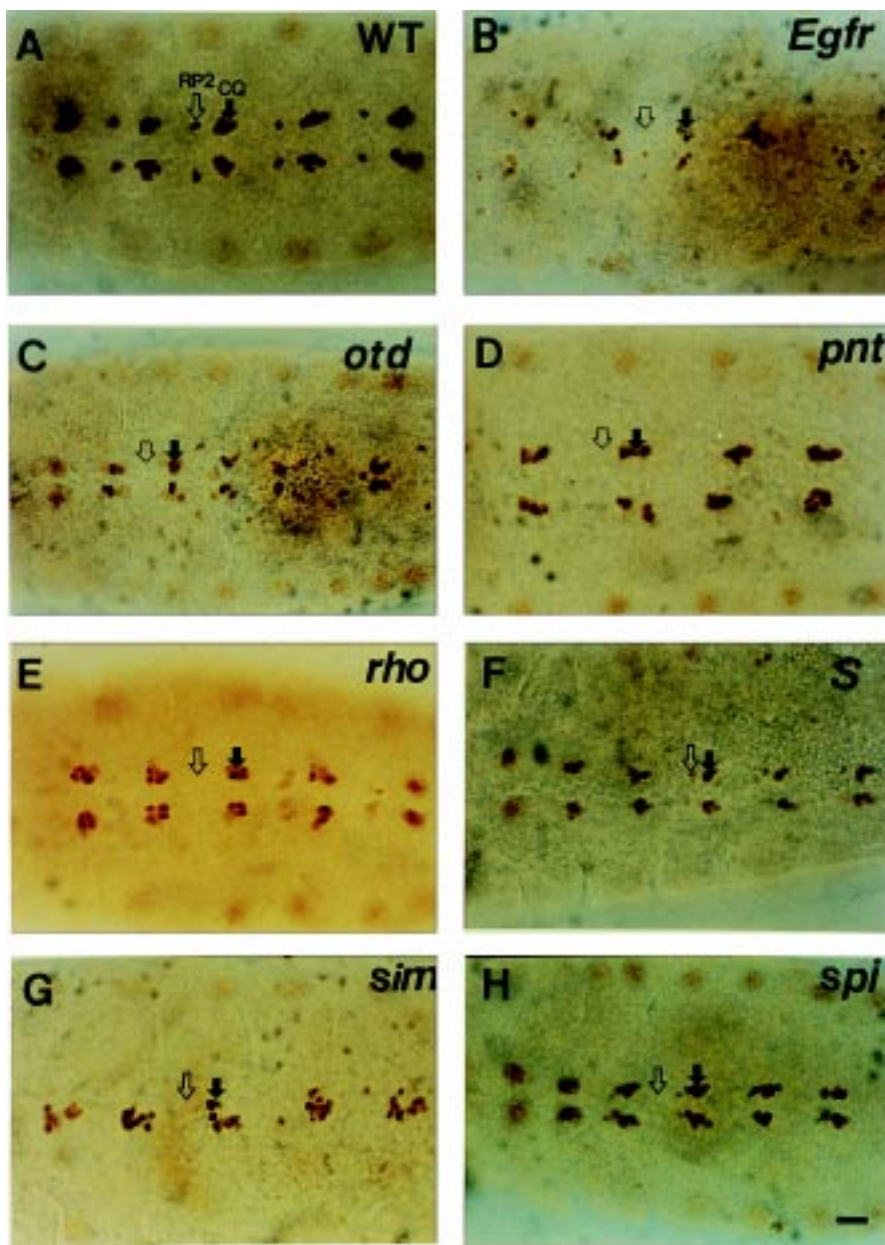


Fig. 6. The CNS midline cells are also required for the formation of the GMCs and neurons during mid-stage neurogenesis. *eve* expression of stage 11 (A) wild-type, (B) *Egfr*, (C) *otd*, (D) *pnt*, (E) *rho*, (F) *S*, (G) *sim*, (H) *spi* in the ventral side of ventral nerve cord is shown. (A) In the wild-type embryo, *eve* is expressed in the RP2 (open arrow) and CQ neurons (filled arrow) per segment. (G) In the *sim* embryo, *eve* expression in more than 95% of RP2 neurons is not shown. The aCC, pCC and CQ neurons become fused at the midline but the number of each neuron is almost the same as that of the wild-type embryo. (B,C,D,E,F,H) In the other *spitz* class mutant embryos, *eve* expression in the RP2 neurons is absent in some of the segments, but the number of *eve*-positive CQ neurons becomes much reduced compared to that of the wild-type embryo. All panels show the ventral view, and anterior is to the left. Bar, 22.5 μ m.

(aCC/ISN), segmental nerve (SN), and RP2/VUM axon pathways in wild-type embryos at stage 15 (Fig. 9A). The SN exits the CNS near the anterior commissures, whereas the ISN originates from the posterior commissures and extends into the adjacent posterior segment of the PNS. In the stage 14 *sim* mutant embryo, the pioneering MP1/dMP2 and pCC/vMP2 axon pathways are fused at the midline, and RP2/VUM axons are absent in more than 90% of hemisegments (Fig. 9B; n=122). Considering that MP1 does not form properly and MP2 identity is changed, and aCC and pCC neurons are still present in *sim* mutant embryos (Figs. 6,7; personal observations), the combined axon pathways may consist of mainly pCC/vMP2 axon pathway. It is possible that defects in identity determination of GMC 4-2 and RP2 neurons observed in the *sim* mutant (Figs. 6,7) lead to the absence of RP2/VUM axon pathway. In the other *spitz* class mutants, the longitudinal MP1/dMP2 pathway is absent in *rho* mutant (Fig. 9E) or disrupted in *pnt*, *S*, and *spi* mutants, and pCC/vMP2 axon pathway shows decreased spacing between longitudinal connectives (Fig. 9C-G). It was also shown that the spacing between anterior and posterior commissures become narrow, and some SNs and RP2/VUM axon pathways are missing. Taken together, decreased spacing between longitudinal connectives that is observed commonly in *spitz* class mutants appears to be mainly due to the misrouting of pCC/vMP2 pathway close to the midline and the disruption or thinning of MP1/dMP2 pathway.

These results indicate that the CNS midline cells and *spitz* class genes also affect proper formation of longitudinal MP1/dMP2 and pCC/vMP2 axon pathways, peripheral SN and RP2/VUM axon pathways during late-stage neurogenesis. It was also demonstrated that the identity change caused by defective *eve* and rG335 expressions in the RP2 neurons of the *sim* mutants may lead to defects in the RP2/VUM axon pathway.

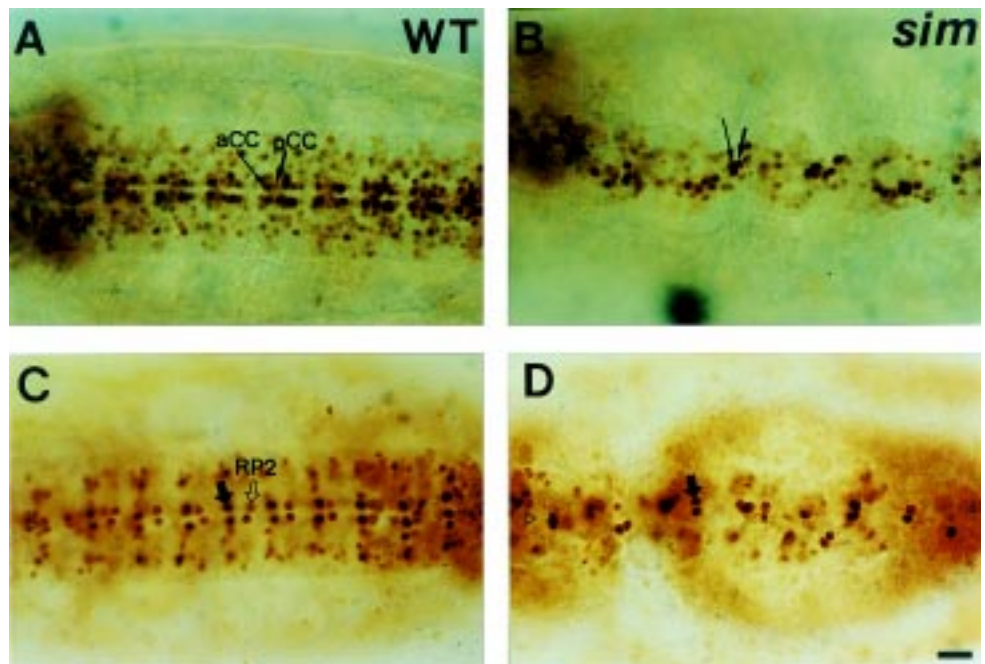
Discussion

It is known that CNS midline cells and *spitz* class genes are required for the proper patterning of the ventral CNS and epidermis (Mayer and Nüsslein-Volhard, 1988; Kim and Crews, 1993; Raz and Shilo, 1993). This study was initiated to carry out a detailed analysis of the effects of CNS midline cells and *spitz* class

genes on the fate determination of NBs and formation of GMCs, neurons, and their axon pathways. The results obtained from this study demonstrate that CNS midline cells and *spitz* class genes are necessary for the initial fate determination of NBs, then formation of a subset of GMCs and neurons, and finally proper formation of the CNS axon pathways at late-stage neurogenesis.

anti-Fas II antibody is known to be suitable for the analysis of axon pathways of the longitudinal connectives (Goodman and Doe, 1993; Lin et al., 1994; Hildago and Brand, 1997). It recognizes the first pioneering longitudinal MP1/dMP2 and pCC/vMP2 axon pathways that are the main components of longitudinal connectives. It also recognizes aCC axons that fasciculate in the intersegmental nerve

Fig. 7. Expression of the aCC/pCC and RP2-specific neuronal markers during mid-stage neurogenesis. (A,B) β -gal expression of A31 enhancer trap line showing the aCC and pCC. At stage 15, aCC (long arrow) and pCC neurons (short arrow) get fused at the midline but the cell number appears to get slightly reduced in *sim* embryos (B). **(C,D)** β -gal expression of rG335 enhancer trap line showing the RP neurons including the RP2 at stage 15. **(C)** The wild-type embryo shows strongly stained RP neurons (filled arrow) and weakly stained RP2 neuron (open arrow). **(D)** In the *sim* mutant, weak β -gal expression in RP2 neurons are missing in at least 80% of the hemisegments. All panels show the ventral view with the ventral midline marked with an open arrowhead, and anterior is to the left. Bar, 22.5 μ m.



The roles of CNS midline cells and spitz class genes in determination of the VNE fate

This study showed that the CNS midline cells are required for proneural gene expression essential for providing competence to generate a NB from equivalent neuroectodermal cells. Of these NBs, NB 7-1 is devoid of *ac* and *en* expression but *ming/lacZ* expression still remains (Figs. 2-4), and the number of aCC, pCC, and CQ neurons derived from the NB 7-1 is almost the same as that of the wild-type embryo when detected with anti-Eve antibody staining and β -gal expression of the specific enhancer trap lines in *sim* embryos (Figs. 6,7). These observations strongly suggest that at least in case of NB 7-1, NB formation and division is not defective but the identity of NB 7-1 has not been correctly assigned. Considering the observation that the *spitz* class mutants show a common phenotype such as the loss of ventralmost epidermis, the involvement of impaired cell division and formation cannot be completely ruled out. However, *eve*-positive aCC, pCC, EL, and CQ GMCs are generated, divide and differentiate into the correct number of neurons in *sim* embryos (Figs. 6,7), indicating that NB formation and division are essentially normal. On the other hand, the loss of specific NB marker gene expressions in the *sim* mutant may well be largely due to the NB identity change. This explanation is supported by the findings that some NB marker expressions are absent but that other marker expressions still remain in a subset of the identified NBs, GMCs and neurons. As described previously, the NB 7-1 shows no *ac* and *en* expression and weak *ming/lacZ* expression during early neurogenesis, indicating that the identity of NB 7-1 has been altered. The absence of *eve* and rG335 expressions in the RP2 neurons in the *sim* mutant may lead to the RP2/VUM axon pathway defect, as detected by the anti-Fas II antibody (Fig. 9). In addition, the loss of *upg/lacZ* expression and the presence of residual *ming/lacZ* expression in the NB 4-1 may cause alteration of NB 4-1 identity, since it is known that NB 4-1 generates the

interneurons that cross anterior and posterior commissures of the CNS (Bossing *et al.*, 1996), and this defect may be in part responsible for the commissural defect observed in the *sim* mutant.

The CNS midline cells may help each NB obtain unique identity and then delaminate from the proneural clusters by sending out signal(s) throughout the VNE. It was reported that EGF receptor signaling pathway is required in the initial patterning of the entire VNE before gastrulation and later in the vicinity of the CNS midline cells during neurogenesis (Skeath, 1998; Udolph *et al.*, 1998; Yagi *et al.*, 1998). It was also shown that Rho and Vein expressed in the VNE activate *Egfr* during stage 5-6 before gastrulation. They were shown to play important roles in the specification and formation of medial and intermediate NBs by repression of proneural gene expression and promotion of NB formation in the intermediate column. In addition, they are also independently involved in the regulation of separate sets of genes, *esg* and *msh* that help medial and lateral NBs establish their unique identity, finally resulting in patterning of medial, intermediate, and lateral columns of the VNE along the D-V axis. Considering that the CNS midline cells are required for the proneural gene expression during the second stage of neurogenesis from this study, they may replace the roles of early-acting genes such as *rho* and *vein* by providing the VNE with additional ligand(s) including the secreted Spi that activates the *Egfr* in the second stage. This may provide the medial, intermediate and lateral NBs with additional positional information to generate diversified NB lineage from the VNE. It was reported that *ac* and *sc* expression in the VNE is necessary for the determination of NB identity as well as NB formation (Parras *et al.*, 1996; Skeath and Doe, 1996), supporting the idea that the CNS midline cells may provide some signal(s) to generate the NBs that have unique identities along the D-V axis. In contrast to the result obtained with *Egfr* mutants, the CNS midline cells influence the proneural gene expression even in the

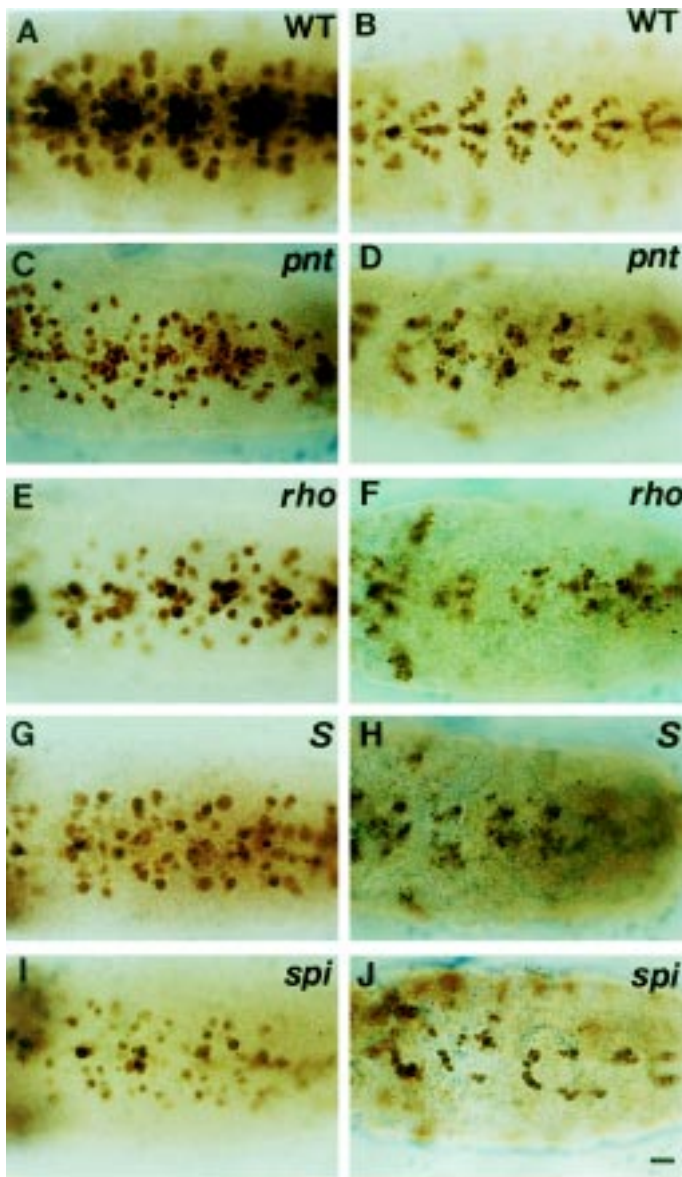


Fig. 8. The *spitz* class genes are required for the proper expression of the *ming/lacZ* and *upg/lacZ* NB markers. (A) *ming/lacZ* expression in a late stage 11 wild-type embryo. (B) *upg/lacZ* expression in a late stage 11 wild-type embryo. (C,E,G,I) *ming/lacZ* expression in late stage 11 *spitz* class mutant embryos. *ming/lacZ* expression is absent in the ventral midline and a number of medial and intermediate NBs. S3 NB 6-1 tends to get fused at the midline, and many NBs become disorganized. *spi* (I) and S (G) exhibit more severe reduction in NB number than *pnt* (C) and *rho* (E). (D,F,H,J) *upg/lacZ* expression in late stage 11 *spitz* class mutant embryos is shown. *upg/lacZ* expression is missing in the midline NBs and almost absent in the medial and intermediate NBs 4-1 and 5-3. All panels show the ventral view, and anterior is to the left. Bar, 22.5 μ m.

lateral NBs, indicating that there may be additional signaling pathway involved in patterning of the VNE by the CNS midline cells. Based on these observations, it remains to be resolved whether the CNS midline cells affect simply NB formation and division or NB identity determination and how the CNS midline cells influence the proper patterning of the VNE.

The possible roles of the CNS midline cells and *spitz* class genes in generating NBs and VE from the VNE

It was shown that the *spitz* class mutants exhibit parallel defects both in the CNS and VE: the absence of ventralmost VE and the reduction in the spacing of the longitudinal connectives and anterior and posterior commissures (Mayer and Nüsslein-Volhard, 1988). Recent molecular genetic and cellular studies have demonstrated that the CNS midline cells and *spitz* class genes are essential for fate determination, division, and differentiation of VE and ventral neurons (Kim and Crews, 1993; Raz and Shilo, 1993; Menne *et al.*, 1997). These results suggest that the *spitz* class genes act in the ventral neuroectodermal precursor cells during early neurogenesis, while the defects in midline glia cell determination and differentiation observed in the *spitz* class mutants (Klämbt *et al.*, 1991; Sonnenfeld and Jacobs, 1994) may be due to more restricted roles of the *spitz* class genes at the later stage of neurogenesis. Since the *spitz* class genes, *Egfr*, and *otd* are known to be expressed in the VNE before and during the period of NB formation, it is possible that the *spitz* class genes divide the overlying NBs into similar regions and provide the NBs with positional information along the D-V axis, just as they divide VE into many specific regions and provide positional information. This would result in parallel defects in the NB and VE in the early stage of neurogenesis. On the other hand, the *spitz* class genes may have additional, more restricted roles in the midline glia differentiation and axon pathway formation during late neurogenesis. This would result in more specific defects in the CNS that are not simply parallel to those observed in the VE such as decreased spacing between longitudinal connectives and fused anterior and posterior commissures. The results obtained from this study appear to support the idea that the *spitz* class genes play homologous roles in the patterning of NBs and VE at least in the early stage of neurogenesis, since the defects in the formation of a subset of NBs and neurons in the *spitz* class mutants are localized mainly medial and intermediate to the CNS midline cells. These results are consistent with previous studies on fate determination of VE showing the abolished expression of VE markers in the ventralmost epidermis (Kim and Crews, 1993; Raz and Shilo, 1993). However, the absence of proneural gene *ac* expression in both medial and lateral NBs and of *ming/lacZ* expression in the lateral NB indicates that there may be distinctive roles of the CNS midline cells and *spitz* class genes in patterning of NBs from the VNE that are different from those in VE patterning.

In conclusion, this study demonstrated that the CNS midline cells are necessary for the fate determination of a subset of NBs and formation of GMCs, neurons, and their axon pathways during *Drosophila* embryonic neurogenesis. The *spitz* class genes also play important roles in establishing NB identity and axon pathway formation. The parallel phenotypes in the CNS and VE in the *spitz* class mutants suggest that homologous functions are responsible for providing the VNE with unique identity along the D-V axis. This study may help understand the basic principles for genesis of neural diversity and assembly of intricate cellular networks with an outstanding degree of specificity in the CNS.

Materials and Methods

Drosophila strains and neural markers

The *Drosophila* strains used in this work were generally the most severe alleles available. The mutant strains used were as follows: *Egfr*^{Δ2},

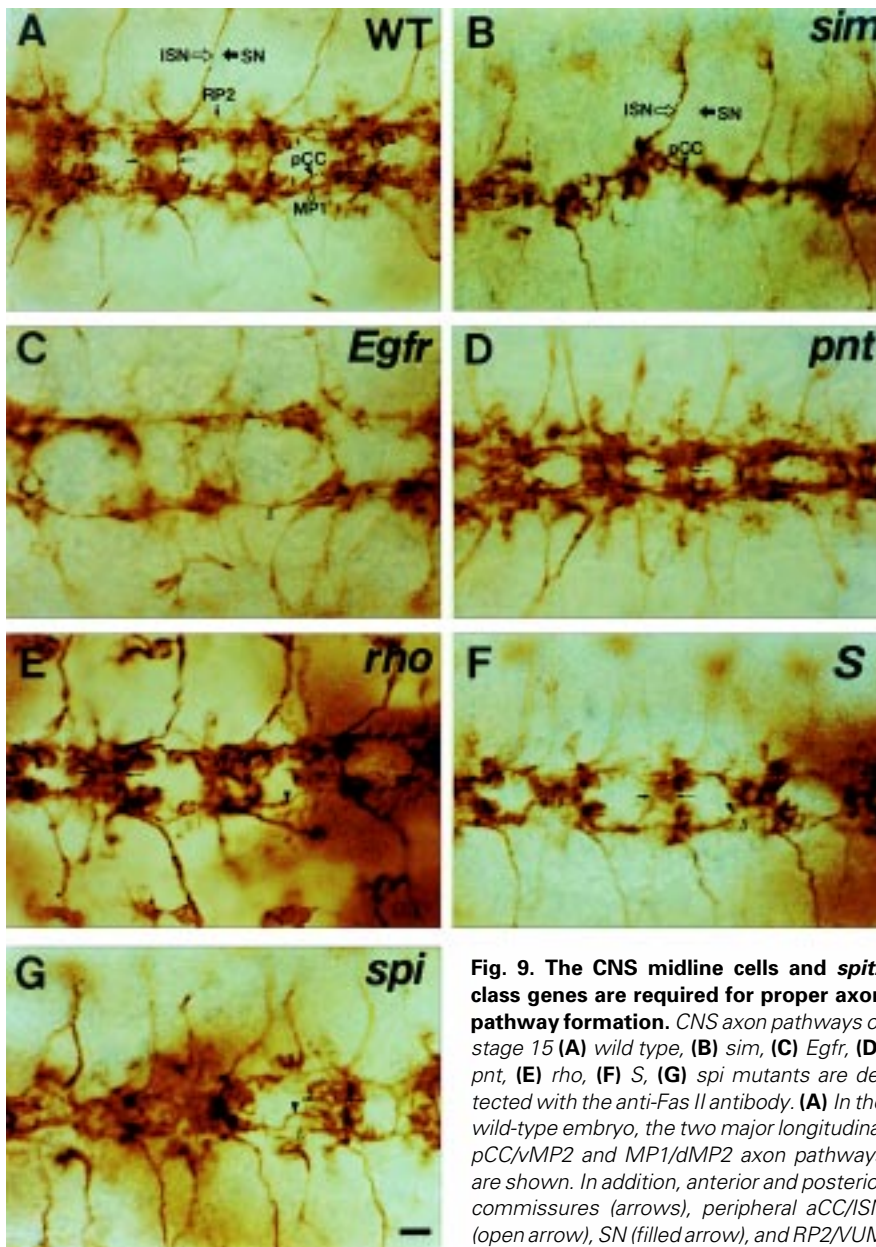


Fig. 9. The CNS midline cells and *spitz* class genes are required for proper axon pathway formation. CNS axon pathways of stage 15 (A) wild type, (B) *sim*, (C) *Egfr*, (D) *pnt*, (E) *rho*, (F) *S*, (G) *spi* mutants are detected with the anti-Fas II antibody. (A) In the wild-type embryo, the two major longitudinal pCC/dMP2 and MP1/dMP2 axon pathways are shown. In addition, anterior and posterior commissures (arrows), peripheral aCC/ISN (open arrow), SN (filled arrow), and RP2/VUM (arrow) motoneuron axon pathways are also

detected. (B) In *sim* embryos, the longitudinal connectives are fused at the midline, indicating that the MP1/dMP2 pathway is missing. RP2/VUM and SN motoneuron axon pathways are missing in 90% of the hemisegments. (C-G) In *spitz* class mutants, the spacing between the pCC pathways is reduced, and the MP1 pathways are disrupted or absent in all of the *spitz* class mutants. The SN and RP2/VUM axons are missing especially in *Egfr* (C) and *rho* (E) mutants. The spacing between anterior and posterior commissures is much reduced. All panels show the ventral view, and anterior is to the left. Bar, 22.5 μ m.

otd⁹, *pnt¹*, *rho⁴*, *S^{IN}*, *sim²* and *spi¹*. NB molecular markers, *ming/lacZ* and *unplugged (upg)/lacZ* (Doe, 1992), kindly provided by Dr. C. Doe, were used to analyze the pattern of NB formation during the S3-S5 stages of neurogenesis. The *ming/lacZ* line contains an enhancer trap P element/*lacZ* insert in the *ming* locus at 83°C on the third chromosome (Cui and Doe, 1992) and the *upg/lacZ* line contains the locus at 44B on the second chromosome (Chiang *et al.*, 1995). The enhancer trap lines AA31 (Grenningloh *et al.*, 1991) and rG335 (C.S. Goodman, unpublished data) were adopted to follow the aCC/pCC and RP neurons, respectively.

Immunohistochemistry and *in situ* hybridization

Embryos collected from genetic crosses between the *spitz* class mutants and neural markers were stained with monoclonal anti- β -galactosidase (β -gal) antibody (Promega) at 1:2000 dilution and detected by horseradish peroxidase immunohistochemistry according to Nambu *et al.* (1991). Homozygous mutant embryos were identified by the lack of expression of a P[*ry⁺*, *ftz/lacZ*] or P[*ry⁺*, *elav/lacZ*] from the second and third balancer chromosomes. Mouse monoclonal anti-engrailed (*en*) antibody 4D9 and anti-Even-skipped (*Eve*) antibody 3C10 (Patel *et al.*, 1989), kindly provided by Dr. N. Patel, were used at 1:20 dilution. Rabbit anti-*Eve* antibody (Frasch *et al.*, 1987), kindly provided by Dr. M. Frasch, was diluted at 1:5000. Mouse monoclonal anti-Fasciclin (*Fas*) II antibody (Grenningloh *et al.*, 1991), kindly provided by Dr. C.S. Goodman, was used at 1:200 dilution. Whole-mount *in situ* hybridization using digoxigenin-labeled DNA probe was performed as described by Tautz and Pfeifle (1989). Whole-mounted embryos were viewed and photographed using an Olympus microscope with Normaski optics.

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