Regulated *vnd* Expression Is Required for Both Neural and Glial Specification in *Drosophila*

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ABSTRACT: The *Drosophila* embryonic CNS arises from the neuroectoderm, which is divided along the dorsal-ventral axis into two halves by specialized mesectodermal cells at the ventral midline. The neuroectoderm is in turn divided into three longitudinal stripes—ventral, intermediate, and lateral. The *ventral nervous system defective,* or *vnd,* homeobox gene is expressed from cellularization throughout early neural development in ventral neuroectodermal cells, neuroblasts, and ganglion mother cells, and later in an unrelated pattern in neurons. Here, in the context of the dorsal-ventral location of precursor cells, we reassess the *vnd* loss- and gain-of-function CNS phenotypes using cell specific markers. We find that over expression of *vnd* causes significantly more profound effects on CNS cell specification than *vnd* loss. The CNS defects seen in *vnd* mutants are partly caused by loss of progeny of ventral neuroblasts—the commissures are fused and the longitudinal connectives are aberrantly positioned close to the ventral midline. The commissural *vnd* phenotype is associated with defects in cells that arise from the mesectoderm, where the VUM neurons have pathfinding defects, the MP1 neurons are mis-specified, and the midline glia are reduced in number. *vnd* over expression results in the mis-specification of progeny arising from all regions of the neuroectoderm, including the ventral neuroblasts that normally express the gene. The CNS of embryos that over express *vnd* is highly disrupted, with weak longitudinal connectives that are placed too far from the ventral midline and severely reduced commissural formation. The commissural defects seen in *vnd* gain-of-function mutants correlate with midline glial defects, whereas the mislocalization of interneurons coincides with longitudinal glial mis-specification. Thus, *Drosophila* neural and glial specification requires that *vnd* expression by tightly regulated. © 2002 John Wiley & Sons, Inc. J Neurobiol 50: 118–136, 2002; DOI 10.1002/neu.10022

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INTRODUCTION

Central nervous system (CNS) specification is a highly intricate task accomplished by the sequential designation of progenitor cells and their progeny. In both invertebrates and vertebrates, conserved hierarchical interactions of key regulators act to restrict the potential of individual cells, or groups thereof, in a spatio-temporal context. In *Drosophila,* the embryonic CNS is generated from bilateral stripes of neuroectodermal cells that are juxtaposed at either side of the ventral midline cells following gastrulation. A subset of these neuroectodermal cells segregates to a subepidermal location to form neuroblasts. Neuroblasts are born in waves that are referred to as S1–S5. The timing of neuroblast birth and the positions neuroblasts assume are relatively invariant (Campos-Ortega and Hartenstein, 1985; Doe, 1992). With each cell cycle the neuroblast buds off a ganglion mother cell (GMC), which divides once to generate a pair of sibling neurons (for review see Campos-Ortega, 1995). At the ventral midline, the mesectodermal cells generate highly specialized neurons and glia (for review see Crews, 1998; Jacobs, 2000).

Dorsal-ventral (D-V) and anterior-posterior (A-P) pattern genes subdivide the neuroectoderm into lon-

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gitudinal and horizontal stripes (for references and review see Skeath, 1999; Cornell and Von Ohlen, 2000). This causes the stereotypical regionalization of the early CNS, with neuroblasts positioned in three longitudinal columns along the D-V axis and in seven rows along the A-P axis. The restricted expression domains of the three homeobox genes, *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*), and *muscle specific homeobox* gene (*msh*), in precise bilateral columns of neuroectodermal cells (Isshiki et al., 1997; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998) correspond to the D-V columnar subdivision of the *Drosophila* neuroectoderm. *vnd* is expressed in ventral neuroectodermal cells (Jimenez et al., 1995; Mellerick and Nirenberg, 1995), while *ind* expression is restricted to intermediate neuroectodermal cells (Weiss et al., 1998), and *msh* is expressed in lateral neuroectodermal cells (D'Alessio and Frasch, 1996; Isshiki et al., 1997). These D-V stripes of neuroectodermal cells in turn give rise to the three columns of neuroblasts—medial or ventral, intermediate, and lateral, respectively. Recent mutant analyses showed that *vnd, ind,* and *msh* are critical for the specification of the ventral, intermediate, and lateral neuroblasts, respectively (McDonald et al., 1998; Chu et al., 1998; Weiss et al., 1998; Buescher and Chia, 1997; Isshiki et al., 1997). Separating the ventral neuroectodermal cells, the ventral midline mesectodermal cells are specified by the transcription factor Single-minded (Sim). In some, but not all, respects the *Drosophila* CNS midline is functionally equivalent to the floorplate of vertebrates (for review and references see Crews, 1998; Jacobs, 2000).

The *NK-2* type homeobox gene, *vnd,* is unique amongst previously described CNS-specific regulatory genes, because it is continuously expressed, from cellularization until the completion of embryonic development, within the developing CNS. *vnd* is expressed in ventral neuroectodermal cells and then in neuroblasts, as well as in GMCs, from the onset of cellularization until the completion of neuroblast delamination at stage 11. Later, the gene is widely expressed in neurons in a pattern relatively unrelated to the early expression pattern (Jimenez et al., 1995; Mellerick and Nirenberg, 1995). *vnd* is essential both for the formation and identity of ventral neuroblasts. In *vnd* mutants the early S1 ventral MP2 and 7.1 neuroblasts are generally not formed (Skeath et al., 1994; Chu et al., 1998; McDonald et al., 1998), while the other surviving early ventral neuroblasts are misspecified, so they assume the identity of their intermediate counterparts. Conversely, over expression of *vnd* leads to a transformation in the identity of intermediate neuroectodermal cells to that of their ventral analogues. In addition, there is a partial transformation in the identity of lateral stem cells to that of their ventral counterparts. Disturbances in the normal *vnd* expression pattern result in the mis-specification of Even-skipped (Eve)-expressing GMCs and neurons (Chu et al., 1998; McDonald et al., 1998). The patterning changes observed in *vnd* loss- and gain-of-function mutants correlate with *vnd's* capacity to repress *ind* and *msh* expression in ventral cells (McDonald et al., 1998) and *ind's* repression of *msh* expression in intermediate cells (Weiss et al., 1998).

In this article we examine the effects of *vnd* mutation and over expression on CNS specification with respect to cell identity. Neurons and glia were examined in the context of their origin from specific D-V columns of precursor cells. We show that neurons that arise from ventral neuroblasts are obliterated in *vnd* embryos. In addition, neurons that arise from the ventral midline are affected when *vnd* is missing in a non-cell autonomous manner. Moreover, RP2, which is derived from the intermediate neuroectoderm, is also nonautonomously affected. Ectopic *vnd* expression results in a variety of effects, including the aberrant positioning of longitudinal connectives too far from the ventral midline. The abnormal location of the interneurons in *vnd* gain-of-function mutants is related to the mis-specification of the longitudinal glia that arise from the lateral neuroectoderm. The behavior and/or presence of commissural neurons are also affected by *vnd* over expression. A lower frequency of midline crossing is observed when *vnd* is over expressed, which correlates with the mis-specification of midline glia. Thus, ectopic expression of *vnd* causes the mis-specification of neurons and glia that arise from all D-V columns of neuroblasts. The implications of these findings are discussed.

METHODS

Drosophila Strains, Heat Shock, and UAS-*Gal4* Transgenic Lines

The *vnd* 6 allele (Jimenez and Campos-Ortega, 1990) was used for loss-of-function analysis. The *HS-vnd* lines, where the p*HS*BJ-Casper vector (Jones and McGinnis, 1993) was used to over express *vnd,* have previously been described (McDonald et al., 1998). *vnd* was ectopically expressed throughout the embryo in the *HS-vnd* lines by collecting embryos from four independent lines after a 1 h laying. Embryos were then aged until they were 3 h old and then heat shocked on coverslips for 7 min at 36°C in 70%

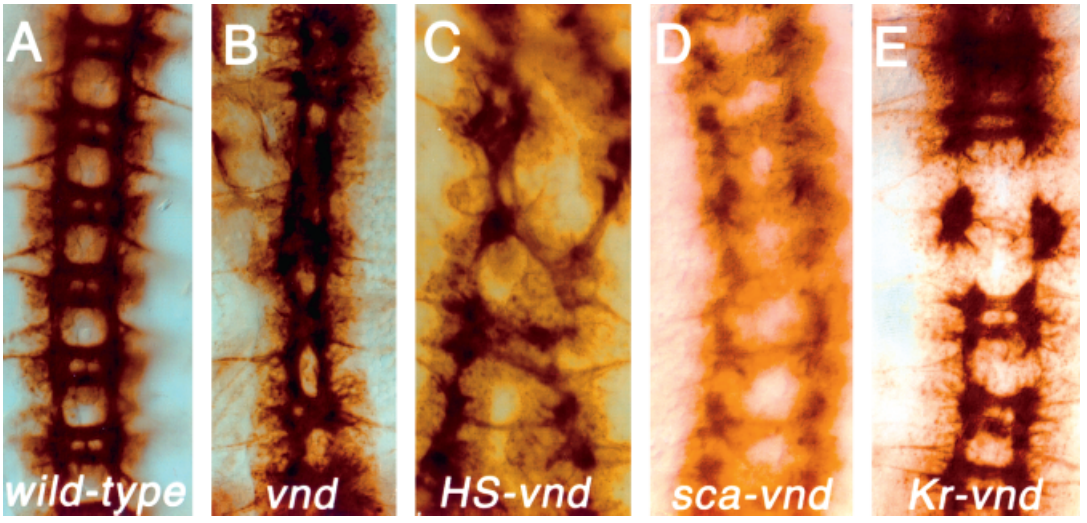

Figure 1 CNS defects in embryos that lack or over express *vnd.* The dissected CNS of BP102-stained embryos is shown; anterior is up. (A) In wild-type embryos BP102 stains the major axons of the CNS, including the bilateral longitudinal connectives, the anterior and posterior commissures, the VUM fascicle extending between the commissures, the ISN, and segmental nerves. (B) In *vnd* embryos the longitudinal fascicles are abnormally close, while the commissures are fused and irregular. The number of motor neurons is reduced. (C) In *HS-vnd* embryos the longitudinal connectives are reduced to clumps of neurons that are irregularly spaced and abnormally distant from the ventral midline. Some, but very few, fascicles cross the midline. (D) The CNS of progeny of UAS-*vnd* X *Gal4-sca* embryos. Although the longitudinal tracts are formed in "*sca-vnd*" embryos, they are discontinuous. In addition, the longitudinal connectives are located further from the midline than in wild-type embryos. Commissural formation is also affected. (E) The CNS of progeny of UAS-*vnd* X *Gal4-Kr* embryos. In "*Kr-vnd*" embryos the CNS phenotype is variable. Generally, in the posterior thoracic segments and the anterior abdominal segments the longitudinal neurons are abnormally positioned too far from the midline. Some neuromeres have a weak to normal phenotype, while others have a more severe one. The intactness of the connectives also varies.

glycerol in PBS. Following a 90 min recovery, another heat shock was administered. The recovery and heat shock was repeated a third time. Then the embryos were aged at 18°C on grape juice agar and were fixed 12–15 h later.

The UAS-*Gal4* system (Brand and Perrimon, 1993) was used to over express *vnd* in spatio-temporally restricted patterns. The UAS-*vnd* construct contained the *vnd* open reading frame and 10 bp of 5′ and 257 bp of 3′ untranslated sequence that was generated by partial EcoRV digestion. The partial cDNA was cloned into the UAS vector using standard procedures. Transgenic lines were generated by microinjection of *white* embryos with the pPi25.7 *wc*DNA according to Spradling (1986). Multiple independent viable lines were generated. Ectopic *vnd* expression was induced by crossing UAS-*vnd* to either *scabrous (sca) Gal4* or *Kruppel (Kr) Gal4,* which were kindly provided by Chris Doe. Oregon R and *white* embryos were used as wild-type controls.

Antibody Staining and Microscopy

The following primary antibodies were used: mouse anti-BP102, 1:10 (Patel, 1994); mouse anti-22C10, 1:10 (Goodman et al., 1984); mouse anti-Engrailed, 1:5 (Patel et al., 1989); rat anti-Even-skipped, 1:2000 (Frasch et al., 1987); mouse anti-Fasciclin II, 1:10 (Van Vactor et al., 1993; Patel, 1994); mouse anti-Fasciclin III, 1:10 (Patel, 1994); rat anti-Single-minded, 1:200 (Ward et al., 1998). A rabbit anti-beta galactosidase (1:2000, Cappel) was used to distinguish homozygous *vnd* embryos. The Vectastain Standard kit (Vector Labs) with a biotinylated secondary antibody was used to detect primary antibody binding. All primary and secondary antibodies were preabsorbed against whole embryos. Antibody binding was detected using nickel enhanced DAB (Pierce) or AEC (Pierce). Embryo dissections, microscopy, and photography were performed as described in Mellerick et al. (1992).

Table 1 Differential Expression of Antigens on Pioneer Neurons Facilitates Their Identification

Neuron	Expresses	Derives from	Reference
MP1	Fasciclin (Fas II), 22C10	Ventral midline	Grenningloh et al., 1991 Klambt et al., 1991 Bossing and Technau, 1994
aCC	Even-skipped (Eve), 22C10, Fas II	1.1 ventral neuroblast	Patel et al., 1989 Broadus et al., 1995 Landgraf et al., 1997
pCC	Eve, Fas II	1.1 ventral neuroblast	Grenningloh et al., 1991 Broadus et al., 1995
SP1	22C10, Fas II	?	Grenningloh et al., 1991
vMP2	22C10, Fas II	MP2 ventral neuroblast	Spana et al., 1995 Grenningloh et al., 1991
dMP2	Fas II	MP2 ventral neuroblast	Spana et al., 1995 Grenningloh et al., 1991
VUM	22C10	Posterior midline neuroblast	Klambt et al., 1991 Goodman et al., 1984 Schmid et al., 1999
U and CQ	Eve	7.1 ventral neuroblast	Patel et al., 1989 Broadus et al., 1995
RP1 and RP3	Fas III	3.1 ventral neuroblast	Patel et al., 1987 Bossing et al., 1996
RP2	Eve, 22C10, Fas II and Fas III	4.2 intermediate neuroblast	Broadus et al., 1995 Bossing et al., 1996
EL	Eve	3.3 lateral neuroblast	Higashijima et al., 1996

RESULTS

Severe CNS Phenotypes Result from *vnd* Mutation or Over Expression

The *Drosophila* embryonic CNS is organized in a simple ladder-like pattern. Two segmental commissures connect the hemi-segments along the mediolateral axis and two longitudinal connectives connect individual neuromeres along the anterior-posterior axis. Figure 1 compares the CNS from a wild-type embryo [Fig. 1(A)] and those from *vnd* loss-of-function [Fig. 1(B); "*vnd* embryos"] and *vnd* gain-of-function embryos stained with the BP102 antibody [Patel et al., 1989; Fig. 1(C–E)]. The CNS of *vnd* embryos is collapsed [Fig. 1(B)], in contrast to the orderly ladderlike scaffold with motor axons emerging laterally seen in wild-type embryos [Fig. 1(A)]. Although the longitudinal connectives are generally formed in *vnd* mutants, they lie too close to the midline. The commissures are poorly formed and fused. The number of motor axons exiting the CNS is significantly reduced [Fig. 1(B)].

We examined the effects of over expressing *vnd* ubiquitously under the control of the heat shock promoter in "*HS-vnd*" embryos. Ectopic *vnd* is easily detected 30 min after a 6–7 min heat shock at 36°C (data not shown). To induce alterations in neuroblast identity *vnd* must be over expressed in neuroectodermal cells prior to their delamination, while *vnd* over expression in delaminated neuroblasts generates little or no obvious effects on neuroblast identity. Alterations in the identity of GMCs requires *vnd* over expression in neuroblasts as well as in neuroectodermal cells (D. Mellerick, J. McDonald, and C. Doe, unpublished observations). Thus, in addition to its primary role in neuroectodermal D-V patterning (McDonald et al., 1998; Chu et al., 1998), *vnd* likely plays secondary roles in lineage specification. In the *HS-vnd* embryos presented here *vnd* was over expressed by heat shocking embryos at 3, 4.5, and 6 h following deposition, timing that coincides with the pre-S1 (stage 8) to S5 (late stage 11) phases of neuroblast delamination. During this developmental time window GMCs are generated from the S1–S4 neuroblasts (for nomenclature see Campos-Ortega and Hartenstein, 1985; Doe, 1992).

Ubiquitous over expression of *vnd* in *HS-vnd* embryos yields an extreme CNS phenotype. The longitudinal fascicles are reduced to clumps of neurons that make minimal contact with one another and are found at irregular distances, too far from the midline. The commissures generally do not form [Fig. 1(C)]. To facilitate interpretation of the *HS-vnd* CNS phenotype, we used the UAS-*Gal4* system (Brand and Per-

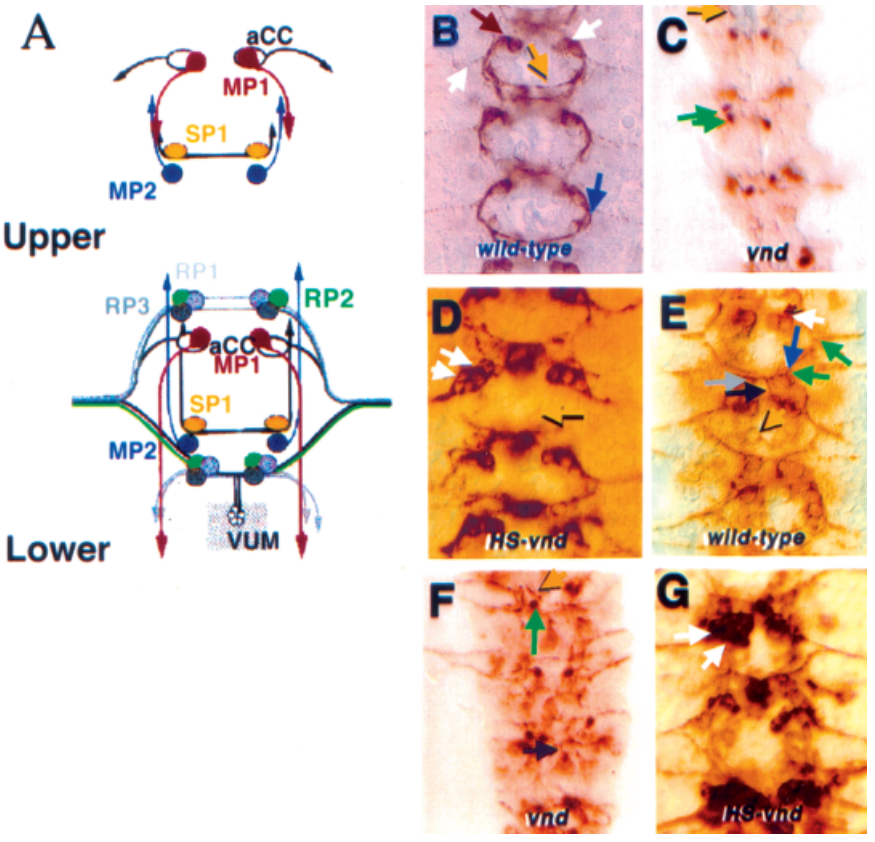

Figure 2 The distribution of 22C10-reactive neurons is altered in embryos that lack or over express *vnd.* (A) Schematic representation of the 22C10-positive neurons and their axonal trajectories detected in a segment of a stage 12.1 wild-type embryo (upper) and a stage 14 embryo (lower). The first 22C10-reactive neurons formed are the MP1 neurons (in red), the aCC motor neurons (in white), and the vMP2 neurons (in blue). The MP1 axons extend posteriorly to fasciculate with the vMP2 axons, which extend anteriorly. The aCC axons extend laterally to pioneer the intersegmental nerve. The SP1 neurons (yellow) extend their axons contralaterally across the midline. Lower: By stage 14 the MP1, vMP2, and SP1 axons have extended into adjacent segments to pioneer the connectives. The RP1 (light purple) and RP3 (gray) neurons project their axons across the midline towards the contralateral RP1 and RP3 neurons. The adjacent RP2 neuron (green) extends its axon anterolaterally to fasciculate with the aCC motor neuron. The VUM neurons extend their axons (black) anteriorly, bifurcate at the RP1 and RP3 commissural fascicles, and then project anterolaterally along the RP2 pathway to fasciculate with the aCC motor neuron. (B) through (G) show ventral views of 22C10-stained embryos. Three to four segments are shown. (B–D) are stage 12.1 embryos, while (E–G) are stage 14 embryos. Embryos in (C) and (F) were also stained for expression of Even-skipped (red stain). (B) Cell bodies and axons in a stage 12.1 wild-type embryo are shown. The cell bodies and axons are indicated with arrows using the color scheme shown in (A). (C) In *vnd* embryos, double stained for 22C10 (brown) and Eve nuclear expression (red), the SP1 neurons express 22C10 (yellow arrow). However, the cell bodies are located abnormally close to the midline. The RP2 neurons are often mislocated and/or duplicated (orange arrows). aCC, MP1, and vMP2 do not express 22C10. (D) In *HS-vnd* embryos an excess of aCC-type motor neurons (white arrows) is detected, and they extend their axons laterally. The cell bodies are located abnormally far from the ventral midline. The SP1 and vMP2 neurons often do not express 22C10 (yellow arrow). (E) The wild-type pattern of 22C10-stained neurons of a stage 14 embryo is shown. Neurons are indicated with arrows using the color scheme shown in (A) (lower). (F) The pattern of 22C10-stained neurons in a stage 14 *vnd* embryo that has also been stained for Eve expression (red stain) is shown. Both the VUM axons (black arrow) and the RP2 axons (green arrow) navigate abnormally. Instead of extending anterolaterally towards the anterior of the hemi-segment, these axons form an abnormally positioned motor fascicle that exits the CNS in the middle of the segment. Only the VUM, SP1, and RP2 neurons express 22C10. (G) In *HS-vnd* embryos the 22C10 staining pattern is variable. In the example shown, large clusters of motor neurons are detected where aCC is normally found (white arrows). 22C10 expression on commissural neurons is reduced.

rimon, 1993) to over express *vnd* in spatio-temporal restricted patterns. Over expression of *vnd* using the *sca Gal4* and the *Kr Gal4* drivers generated CNS phenotypes that were more subtle than that seen in *HS-vnd* embryos, yet similarly led to embryonic lethality. *Sca Gal4* directs expression continuously throughout neurogenesis, firstly in most neuroectodermal cells, later in neuroblasts and GMCs, and then in neurons (Mlodzik et al., 1990). The longitudinal connectives are discontinuous and positioned farther from the midline than usual in embryos over expressing *vnd* under the control of the *sca* driver (in "*sca-vnd*" embryos). Commissural formation is also affected [Fig. 1(C)]. Over expression of *vnd* in the *Kr* domain (in "*Kr-vnd*" embryos) leads to a CNS phenotype that is more variable than in *HS-vnd* or *sca-vnd* embryos, as shown in Figure 1(E). *Kr Gal4* directs gene expression in a gap gene pattern from cellularization onwards in the T2-A4 parasegments (Gaul et al., 1987).

Thus, *vnd* embryos have collapsed longitudinal connectives and fused commissures, whereas *vnd* over expression causes the dorsal mislocalization of the longitudinal connectives and a reduction in commissure formation.

Pioneer Neurons Are Mis-Specified When *vnd* Expression Is Perturbed

The early forming neuroblasts that are mis-specified in *vnd* loss- and gain-of-function mutants (McDonald et al., 1998; Chu et al., 1998) produce the pioneer neurons (Bate, 1976) that form the axonal scaffold onto which later-forming neurons fasciculate (Bossing et al., 1996; Broadus et al., 1995; Schmid et al., 1999). We examined these neurons in *vnd* loss- and gain-of-function mutants for defects in specification and/or numbers using a number of cell-specific markers. Table 1 summarizes the markers examined. The origin of the neurons examined from ventral (*vnd*+), intermediate (*ind*+), and lateral (*msh*+) neuroblasts, as well as the ventral midline (*sim*+), is also indicated.

Initially, we compared the distribution of microtubule associated 1B-like protein, which recognizes the 22C10 antibody, in *vnd* loss- and gain-of-function mutants to that in wild-type embryos, because this antigen is expressed on a number of well characterized pioneer neurons and their axonal projections (Goodman et al., 1984; Hummel et al., 2000). Normally, the ventral-midline derived MP1 neuron (Bossing and Technau, 1994) and the ventral-neuroblast derived vMP2 neuron (Spana et al., 1995) express 22C10 at late stage 12. These neurons are positioned anterolaterally and posterolaterally, respectively, at either side of the ventral midline and project their axons towards each other to fasciculate. About the same time, the anterolaterally positioned aCC motor neuron (which sits dorsal to MP1 and originates from the ventral 1.1 neuroblast; Broadus et al., 1995) projects its axon laterally to pioneer the inter-segmental nerve (ISN). Then, the two SP1 neurons, which are posteriorly located at either side of the ventral midline anterior to the vMP2 neurons, project their axons contralaterally across the midline towards each other. Upon contact with its complimentary SP1 neuron, the SP1 axon extends anteriorly [Grenningloh et al., 1991; Fig. 2(A), upper and 2(B)].

Figure 2(C) shows a stage 12.1 *vnd* embryo that has been double stained for 22C10 (brown) and Eve expression (red). In *vnd* embryos neither aCC, MP1, nor vMP2 express 22C10 [Fig. 2(C)]. Because the 1.1 ventral neuroblast expresses markers characteristic of intermediate row 1 neuroblasts in *vnd* mutants (McDonald et al., 1998; Chu et al., 1998) and the MP2 neuroblast does not form in *vnd* mutants (Skeath et al., 1994), the lack of 22C10 expression in both these neurons is somewhat predictable. The fact that the MP1 neurons does not express 22C10 in *vnd* embryos was unexpected, because this neuron originates from the mesectoderm (Klambt et al., 1991; Bossing and Technau, 1994), where *vnd* is only transiently expressed very early in development (Chu et al., 1998; Jimenez et al., 1995). SP1 forms late in *vnd* embryos. Otherwise this neuron is wild-type, apart from its abnormal location too close to the ventral midline. RP2 is either mislocated or duplicated when *vnd* is mutated [Fig. 2(C), orange arrows].

vnd over expression leads to increased levels of 22C10-expressing neurons in the anterior of the hemisegment, at the expense of 22C10-positive posterior hemi-segment neurons. In late stage 12 *HS-vnd* embryos an excess of 22C10-positive aCC-type neurons is detected in the anterior of the hemi-segment that extend their axons laterally, while the SP1 neuron (whose origin is unknown) often does not express the antigen [Fig. 2(D)]. Unexpectedly, the vMP2 neuron also occasionally fails to express 22C10 when *vnd* is over expressed. vMP2 arises from the MP2 ventral neuroblast (Bossing et al., 1996; Spana et al., 1995) that normally expresses *vnd* (Jimenez et al., 1995). The fact that this neuron is affected suggests that the level of *vnd* expression must be tightly regulated throughout development for neurons derived from ventral neuroblasts to be appropriately specified. 22C10-positive neurons are aberrantly positioned too far from the ventral midline when *vnd* is over expressed. Thus, lack or over expression of *vnd* leads to unexpected neuronal mis-localization.

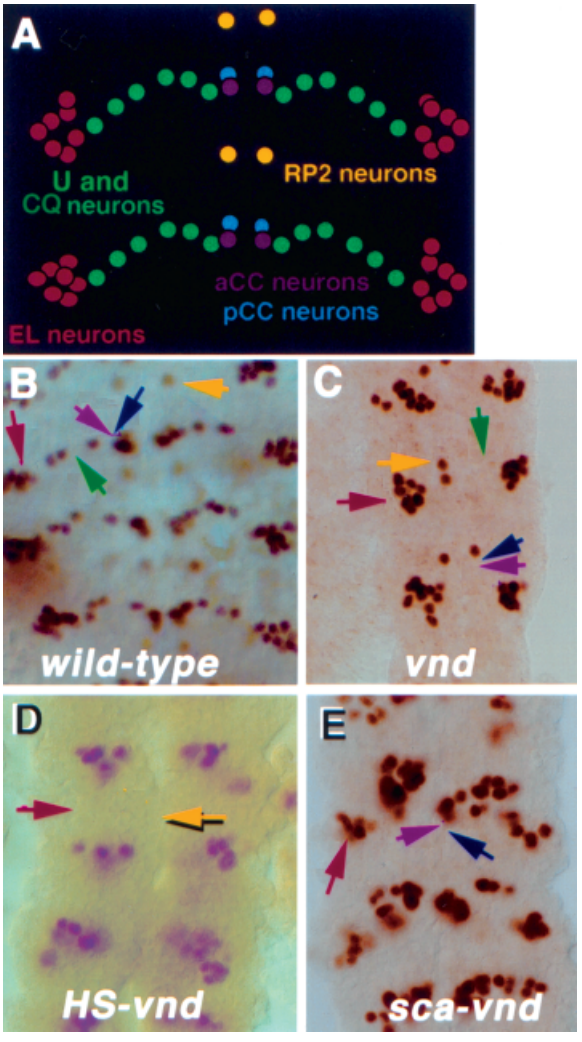

Figure 3 Subsets of Even-skipped expressing neurons are mis-specified when *vnd* expression is altered. (A) and (B) Eve is detected in the laterally positioned EL neurons (red), in the U and CQ neurons (green), in the midline proximal aCC motor neurons (pink), and in the pCC (blue) neurons. In addition, the RP2 (yellow) motor neuron [which is on the dorsal side of the CNS and thus slightly out of focus in (B)] expresses Eve. Note that the U and CQ neurons are located between the midline proximal aCC/pCC neurons and the lateral EL neurons. The color scheme in (A) is used in (B–E) to identify specific Eve-positive neurons. (C) In *vnd* embryos, the aCC/pCC, CQ, and U neurons (green, blue, and pink arrows) do not express Eve, while the RP2 neurons are sometimes duplicated (yellow arrow) and/or inappropriately positioned. (D) In *HS-vnd* embryos clumps of neurons are detected in an antero-midline proximal position. These may be over-specified aCC, pCC, CQ, and/or U neurons. The RP2 neurons are generally missing (yellow arrow), while the expression of Eve in the EL neurons is often reduced. (E) The distribution of Eve in *sca-vnd* embryos parallels that seen in *HS-vnd* embryos, although the Eve-positive EL neurons are generally present.

The altered distribution of 22C10 in older *vnd* embryos reveals a novel aspect of the *vnd* phenotype—non-cell autonomous defects in neurons that are derived from both the ventral midline and the intermediate neuroectoderm. Figure 2(F) is a stage 14 CNS from a *vnd* embryo that was double stained for 22C10 (brown) and Eve expression (red). Only three 22C10-positive neurons are detected—RP2, SP1, and the ventral unpaired midline (VUM) neurons. However, the VUM axonal trajectories are abnormal. In wild-type embryos these neurons project their axons

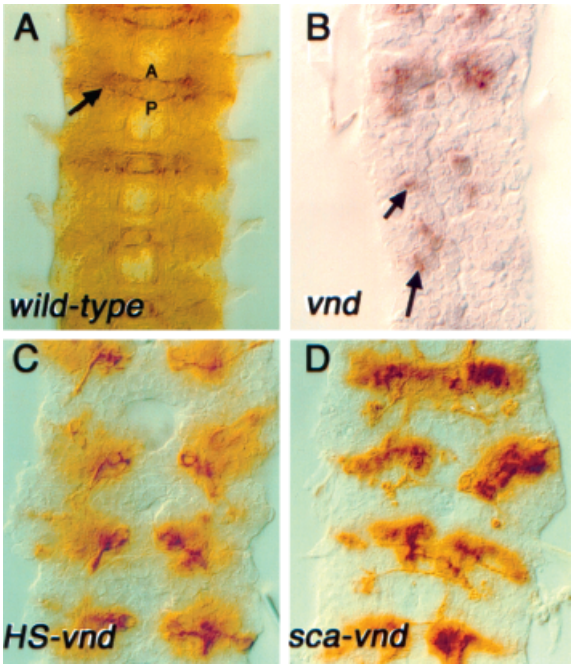

Figure 4 Commissural expression of Fas III is altered in embryos that lack or over express *vnd.* (A) In wild-type stage 14 embryos, Fas III is expressed on the RP1/RP3 sibling neurons, as well as four additional commissural fascicles that make up the anterior (A) and posterior (P) commissures. The RP1/RP3 fascicle expresses Fas III as it extends across the midline. The antigen continues to be expressed as the fascicles extend posteriorly after contact with the contralateral RP1/RP3 neurons. In addition, the RP2 motor neuron expresses the antigen (black arrow). (B) In *vnd* embryos, none of the commissural neurons express Fas III. However, the RP2 cell bodies express the antigen. These cell bodies are often unevenly distributed and/or duplicated (black arrow). (C) In *HS-vnd* embryos, the Fas III positive fascicles often extend their axons away from rather than towards the ventral midline. (D) In *sca-vnd* embryos the pattern of Fas III expression is often irregular. Although some Fas III expressing neurons do extend contralaterally, their location and axonal projections are often disorganized. As seen in *HS-vnd* embryos, some Fas III-expressing axons extend posterolaterally rather than across the midline.

anteriorly, bifurcate at the anterior commissure, fasciculate with RP2 axons, and follow the aCC anterolaterally into the ISN [Fig. 2(A), lower and 2(E)]. However, in *vnd* embryos the VUM axons exit the CNS posterolaterally. RP2 pathfinding is also abnormal in *vnd* embryos. The RP2 axon exits the CNS very near its cell body [Fig. 2(F)], in contrast to the wild-type situation where the axon projects anterolaterally to fasciculate with aCC [Fig. 2(A), lower and 2(E)]. In addition, the VUM and RP2 axons fasciculate at an abnormal position posterolaterally in *vnd* embryos [Fig. 2(F)]. RP2 behaves like aCC when *vnd* is mutated, in terms of the direction in which its axon extends and its axon extends and its fasciculation with the VUM axons laterally. Because RP2 is generated from the intermediate 4.2 neuroblast (Broadus et al., 1995), which does not express *vnd,* we were surprised to find this neuron has pathfinding defects. In *HS-vnd* embryos 22C10-positive neurons at the posterior of the hemi-segment are generally lacking, while clumps of neurons are detected at the position of the aCC neurons [Fig. 2(G)].

Thus, 22C10 staining highlights expected aspects of the *vnd* loss- and gain-of-function phenotypes, including the loss of neurons arising from ventral neuroblasts in *vnd* embryos and their over-specification when *vnd* is ectopically expressed. However, in addition, novel aspects of the phenotypes are apparent, particularly in *vnd* embryos, including non-cell autonomous ventral midline defects, as well as the pathfinding abnormalities of the RP2 and VUM neurons.

Stem Cells Are Recruited to Inappropriate Lineages at the Expense of Alternative Lineages When *vnd* Expression Is Disturbed

The transcription factor, Eve, is widely used to analyze cell fate changes during CNS development. Previous analyses indicated that the distribution of Eve is perturbed in GMCs and neurons that arise from ventral and intermediate neuroblasts when *vnd* is missing or over expressed (McDonald et al., 1998; Chu et al., 1998). Here we reconfirm and extend these analyses. Normally, Eve is expressed in RP2, in the sibling aCC and pCC neurons, in the U and CQ neurons, and in the Eve lateral (EL) neurons (Patel et al., 1989). RP2 arises from the intermediate 4.2 neuroblast, while aCC/pCC arise from the ventral anterior 1.1 neuroblast, and the U and CQ neurons arise from the ventral posterior 7.1 neuroblast. The EL neurons originate from the lateral 3.3 neuroblast [Broadus et al., 1995; Higashijima et al., 1996; Fig. 3(A,B)]. In *vnd* embryos the aCC, pCC, U, and CQ neurons are absent, while the RP2 neurons are sometimes duplicated and/or mislocated [Fig. 3(C)]. Over-expression of *vnd* leads to one or more clumps of neurons at the anterolateral position [Fig. 3(D,E) and data not shown]. Their position is consistent with duplications of aCC, pCC, or U neurons, or some combination of these three. Indeed, 22C10-Eve double staining of *sca-vnd* embryos indicated that many, but not all, of the duplicated Eve-positive neurons in these embryos were aCC-type neurons (data not shown). RP2 neurons are generally missing in *vnd* gain-of-function mutants [Fig. 3(D,E)], although in some instances RP2 neurons are duplicated rather than removed (data not shown). The EL neurons are usually missing in the *HS-vnd* mutants [Fig. 3(D)] but are only moderately affected in *Kr-vnd* and *sca-vnd* embryos [Fig. 3(E) and data not shown]. The effects of *vnd* over expression on the EL neurons have not been described previously.

Loss of Commissural Neurons and Abnormal Pathfinding Contributes to the Commissural Phenotype in *vnd* Loss- and Gain-of-Function Mutants

The commissural phenotypes in *vnd* loss- and gain-of-function mutants could result from either of two defects, both of which would produce a similar phenotype: the lack of commissural neurons or abnormal axonal projections of these neurons. To address these alternatives, we examined the distribution of Fasciclin III (Fas III), which is expressed on multiple cell bodies, as well as regionally on five commissural fascicles. These include the RP1 and RP3 fascicles and a third additional anterior commissural fascicle, as well as two posterior commissural fascicles. RP2 is one of the cell bodies that expresses Fas III [Fig. 4(A), black arrow; Patel et al., 1987]. In *vnd* embryos, although RP2 cell body staining is detected, the cell bodies are often aberrantly located and/or duplicated [black arrow, Fig. 4(B)], and no commissural expression of Fas III is detected. Jimenez and Campos-Ortega (1990) showed that progeny of ventral neuroblasts often die prematurely in *vnd* embryos. Because RP1 and RP3 originate from the ventral 3.1 neuroblast (Bossing et al., 1996), the absence of these Fas III-expressing fascicles was expected. The ventral 2.2, 4.1, and 5.2 neuroblasts generate commissural neurons (Schmid et al., 1999). Potentially, any or all of these neurons could be adversely affected in *vnd* mutants and contribute to the absence of Fas III commissural expression.

Fas III staining of *vnd* gain-of-function mutants showed that both pathfinding defects and reduction

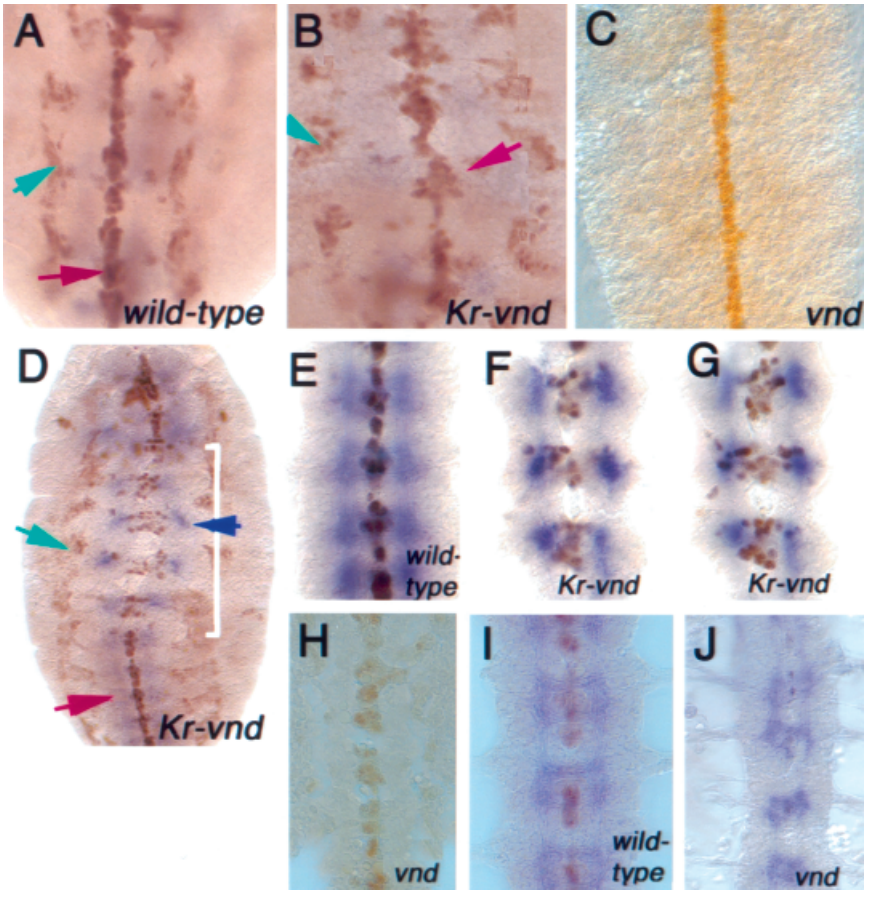

Figure 5 *vnd* over expression results in over specification and mislocalization of midline glia. (A–C) and (H) show Sim distribution in a *Kr-vnd* embryo (A,B) and in *vnd* embryos (C) and (H). (D–G) show Sim expression (brown) and BP102 expression (blue) in *Kr-vnd* embryos, while (I and J) shows Sim (brown) and BP102 (blue) expression in a wild-type (I) and *vnd* embryo (J). Anterior is up. (A) and (B) Dorsal and ventral views of a single stage 12.5 embryo. Sim is expressed in ventral midline mesectodermal cells (red arrow) and in lateral muscle cells (green arrow). (A) In the wild-type domain of this *Kr-vnd* embryo, Sim-expressing mesectodermal cells are organized in a single, typically two cell wide, column at the ventral midline. The cells have lost contact with the ventral surface. (B) Targeted *vnd* over expression leads to an increased number of Sim-expressing cells in the abdominal segments shown. (C) The expression pattern of Sim in the ventral midline is normal in *vnd* embryos, although the protein is not seen in the lateral muscle cells. (D) In the early stage 13 *Kr-vnd* embryo shown, the pattern of Sim-expressing cells is very abnormal. In the thoracic and upper abdominal segments (highlighted with the white bar), the Sim-expressing cells are dispersed over the ventral surface of the developing CNS (blue, see arrow) at a superficial location. This contrasts with the distribution of Sim-expressing cells in the posterior abdominal segments, where they are organized in a two-cell wide column. (E), (F), and (G) Sim expression (brown) and BP102 expression (blue) in a single midstage 13 *Kr-vnd* embryo. (E) The Sim-expressing midline glia (brown) are highly organized between the longitudinal connectives (blue) in the wild-type domain. (F) and (G) Sim-expressing midline "glia" (brown) and the longitudinal connectives (blue) are shown in two different focal planes of the *Kr* domain, where *vnd* over expression has been targeted. An excess number of Sim-expressing nuclei are distributed randomly between, as well as on top of, the lateral neurophiles. Some Sim-expressing nuclei are positioned lateral to the longitudinal connectives. (H) The Sim-expressing midline glia (brown) are relatively normal in a *vnd* embryo that is of similar age to that shown in (E–G). (I) By the time the commissures (blue) have separated in a stage 14 wild-type embryo, the Sim-expressing midline glia are highly organized at the anterior of the segment. (J) In a stage 14 *vnd* embryo the number of midline glia (brown) is significantly reduced and the Sim-positive nuclei are reduced in size. The BP102-expressing neurons are in blue.

in the number of commissural neurons contribute to the phenotype seen. Fas III-expressing commissural fascicles are generally not detected in *HS-vnd* embryos [Fig. 4(C)]. In addition, the number of Fas III-expressing neurons is reduced in *HS-vnd* embryos compared to wild-type embryos. Often, two Fas III-expressing fascicles are detected in each hemi-segment of *HS-vnd* embryos. These axons project posteriolaterally [Fig. 4(C)] instead of across the ventral midline as seen in wild-type embryos [Fig. 4(A)]. In *Kr-vnd* and *sca-vnd* embryos the Fas III phenotype is weaker [Fig. 4(D) and data not shown]. In the *sca-vnd* embryos shown in Figure 4(D), the number of Fas III-expressing neurons is generally greater than that seen in *HS-vnd* embryos. Some Fas III-positive fascicles do extend contralaterally as seen in wild-type embryos. However, their position is often highly irregular. In addition, some neurons that express Fas III extend their axons posterolaterally, instead of across the ventral midline [Fig. 4(D) and data not shown]. Thus, the commissural phenotype seen in *vnd* embryos is partially caused by lack of critical pioneering neurons, whereas that seen in gain-of-function mutants is due to the combined effects of neuronal obliteration and abnormal pathfinding.

Midline Glia Are Affected When *vnd* Expression Is Disturbed

The MP1 neuron, which is derived from the mesectoderm, is either mis-specified or absent in *vnd* loss- and gain-of-function mutants. In addition, contralateral crossing of commissural neurons is affected. Because midline glia provide cues for axon guidance across the midline (for review see Jacobs, 2000), we asked whether these cells are affected in *vnd* loss- and gain-of-function mutants by monitoring the distribution of the transcription factor, SIM. The expression pattern of this key regulator was similar to that in wild-type in both loss- and gain-of-function mutant embryos until stage 12 (data not shown). Thus, *vnd* does not regulate *sim* expression directly. However, as CNS development proceeded further the distribution of SIM was altered in a manner suggesting that midline glial viability or specification is indirectly dependent on *vnd.* Figure 5(C) shows SIM expression in a stage 12 *vnd* loss-of-function embryo. The ventral midline expression of Sim is comparable to the wild-type pattern shown in Figure 5(A). In an early stage 13 embryo the pattern of SIM expression is also comparable to the wild-type pattern [compare Fig. 5(H) and (E)]. However, during stage 13 there is a drastic reduction in the number of SIM-expressing nuclei, and those nuclei that stain are very small relative to those seen in wild-type embryos. This inappropriate loss of *sim* midline glial expression is potentially related to the lack of key commissural neurons (Hummel et al., 1999).

Strikingly, *vnd* over expression led to a profound effect on *Sim* expression. In wild-type embryos, SIM-expressing mesectodermal cells become arranged in two adjacent columns at the midline and lose contact with the ventral surface during stage 12 [Fig. 5(A)]. In contrast, when *vnd* over expression is targeted using *Kr-Gal4,* an increased number of SIM-expressing mesectodermal cells are inappropriately located in an expanded domain [Fig. 5(B)]. In stage 13 *Kr-vnd* embryos the SIM-expressing midline cells (brown) are located on the ventral surface of the embryo between the aberrantly positioned BP102-expressing neurons (blue), and holes have developed at the ventral midline between the SIM-expressing cells [Fig. 5(D)]. Figure 5(E) shows SIM expression in the wild-type domain of a stage 13 *Kr-vnd* embryo; the SIM-expressing mesectodermal cells are highly organized and are becoming restricted to the anterior of the segment [see last five segments in Fig. 5(D)]. Figure 5(F) and (G) shows two focal planes of the same *Kr-vnd* embryo, where ectopic *vnd* expression has been targeted. Clearly more SIM-expressing "glia" are present relative to that seen in the wild-type domain of the same embryo. In addition, the SIM-expressing cells are dispersed in a haphazard fashion and even extend over, and dorsal to, the developing lateral neurophiles. Typically, the number of Sim-expressing midline "glia" is close to twofold higher when *vnd* is over expressed relative to that seen in the wild-type domain.

Thus, midline glial numbers are reduced late in development in *vnd* loss-of-function mutants. Over expression of *vnd* results in an overabundance of Sim-expressing "midline glia" that are aberrantly positioned and dispersed throughout the expanded mesectoderm. Our data indicate that *vnd* does not, however, regulate *sim* expression directly.

Mis-Specification of Both Pioneering Neurons and Longitudinal Glia Causes the Longitudinal Connective Phenotype in *vnd* Gain-of-Function Mutants

The aberrant distribution of the cell markers analyzed thus far only partly explains the longitudinal connective phenotype in *vnd* loss- and gain-of-function mutants. To further elucidate the effects of *vnd* misexpression on the formation of the longitudinal connectives, we examined the distribution of Fasciclin II (Fas II), which is expressed on the MP1 pathway that pioneers these connectives (Grenningloh et al., 1991). Wild-type late stage 12 embryos express

Fas II on the posterior descending fascicle of the MP1/dMP2 neurons and on the anterior extending fascicle of the pCC/vMP2 neurons of the MP1 pathway. In addition, Fas II is expressed on the SP1 fascicle that makes contact with the MP1 pathway and on the RP2 and aCC motor fascicles [Grenningloh et al., 1991; Hidalgo and Brand, 1997; Fig. 6(A,B)].

In *vnd* embryos, RP2, the VUM fascicle, and the SP1 fascicle express Fas II [out of focus Fig. 6(C)], although normally the VUM fascicles do not express this antigen [Fig. 6(A,B) and data not shown]. In agreement with the abnormal RP2 pathfinding detected using the 22C10 antibody [see Fig. 2(C)], the RP2 axons that express Fas II extend posterolaterally [Fig. 6(C)] rather than anterolaterally as seen in wild-type embryos [Fig. 6(A,B)]. In *HS-vnd* embryos the Fas II-expressing neurons are highly disorganized and thus difficult to distinguish [Fig. 6(D)]. In *sca-vnd* embryos the aCC neurons are clearly duplicated. The number of Fas II-positive neurons at the posterior of the hemi-segment is also generally reduced. These include RP2, vMP2, and dMP2 [Fig. 6(E)].

In stage 14 wild-type embryos, Fas II is expressed on two longitudinal fascicles and on the motor tracts of aCC and RP2, as well as on four commissural tracts, including the SP1 fascicle [Fig. 6(F); Grenningloh et al., 1991; Hidalgo and Brand, 1997]. In *vnd* embryos of similar age, expression of Fas II on the ascending SP1 fascicles is irregular [Fig. 6(G)], potentially because the anterior extending pCC/vMP2 fascicle is not formed (Hidalgo and Brand, 1997). In *HS-vnd* embryos the Fas II-expressing longitudinal tracts are discontinuous. In addition, the number of Fas II-expressing neurons decreases with age [compare Fig. 6(D) and (H)]. In *sca-vnd* embryos the phenotype observed is generally similar but weaker [Fig. 6(I)]. In stage 15 wild-type embryos the Fas II-expressing fascicles have become reorganized into three longitudinal tracts—the medial vMP2 fascicle, the more dorsal dMP2/MP1/pCC fascicle, and a third MP fascicle (Hidalgo and Brand, 1997). This pattern is highly disrupted in embryos that over express *vnd* in a dose-dependent manner. In *Kr-vnd* embryos the pattern in the postero-abdominal segments is wild-type [Fig. 6(J)]. However, in the thoracic segments and the antero-abdominal segments the three longitudinal tracts are often highly disrupted [Fig. 6(J,K)].

Over expression of *vnd* leads to a partial transformation in lateral stem cell identity (McDonald et al., 1998; Chu et al., 1998). If the glial progeny of the lateral neuroblasts are mis-specified as a result of *vnd* over

Figure 6 The distribution of Fas II-expressing neurons is altered in embryos that lack or over express *vnd.* (A) Schematic representation of two wild-type segments showing the cell bodies and axons that express Fas II during stage 12. These include the aCC (white) and pCC (navy) neurons in the anterior corner of the hemi-segment. The aCC axon projects posteriolaterally while the pCC axon projects anteriorly. The MP1 axon (red) extends posteriorly to fasciculate with the vMP2 axon (blue). dMP2 (pink), which is positioned dorsal to vMP2, extends its axon posteriorly. RP2 (green) stains weakly and extends its axon anterolaterally (see Fig. 2), while the SP1 axon (yellow) also expresses the antigen weakly. Neurons in (B–K) are identified using the color scheme shown in (A). (B) In stage 12.1 wild-type embryos Fas II is expressed on the neurons of the MP1 pathway, as shown in (A). The red arrow indicates the position of the MP1 cell body in each hemi-segment. (C) In *vnd* embryos of similar age, Fas II expression is primarily restricted to RP2 (green arrows) and the VUM fascicle. Note that the RP2 axons extend slightly posteriorly rather than anterolaterally as seen in wild-type embryos. The SP1 axons also express Fas II (out of focus). (D) In *HS-vnd* embryos of a similar age, the pattern of Fas II-expressing neurons is very disorganized and discontinuous. (E) In *sca-vnd* embryos, clusters of aCC-type neurons are detected (white arrows). Expression of the antigen in the posterior of the hemi-segment is reduced. Fas II expression is reduced in dMP2, vMP2, and RP2 neurons (see yellow and blue arrows). (F) In wild-type stage 13 embryos, two longitudinal fascicles express Fas II, as well as four commissural fascicles, which include the SP1, RP2 (green arrow), and aCC (white arrow) fascicles. (G) In *vnd* embryos of a similar age, a single fascicle is typically detected that expresses Fas II. However, it is discontinuous. This is potentially the anterior ascending SP1 fascicle that expressed the antigen earlier. (H) In stage 13 *HS-vnd* embryos the pattern of fascicles is highly disorganized. The number of Fas II-expressing neurons has decreased in comparison to earlier stages [compare (H) to (D)]. (I) The phenotype in *sca-vnd* embryos is weaker than in *HS-vnd* embryos. (J) Whole mount staining of a stage 15 *Kr-vnd* embryo. In the posterior abdominal segments the Fas II-expressing axons are organized into three parallel fascicles. In the anterior abdominal segments and the posterior thoracic segments the fascicles are interrupted or fused. (K) Four segments of a *Kr-vnd* embryo are shown. All three Fas III-positive fascicles are obliterated in some segments, while the medial one is intact and the two outer ones are abolished in other segments. This phenotype is variable.

expression, this could potentially explain why the longitudinal connectives are inappropriately positioned. Thus, we examined the distribution of the glioblast/glial-specific transcription factor, Reversed Polarity (Repo), to address whether misexpression of *vnd* affects the development of these cells. Repo is expressed in all glial cells and their precursors, apart from the midline glial lineage, from stage 11 to the end of embryonic development (Xiong et al., 1994; Halter et al., 1995). Repo distribution is affected by *vnd* over expression. In *sca-vnd* embryos, expression of Repo in longitudinal glioblasts is generally wild-type (data not shown). However, the number of Repo-positive longitudinal glia is often reduced compared to that in wild-type embryos [compare Fig. 7(A) and (C)]. In addition, in contrast to the migration of wild-type longitudinal glia to a mediolateral position, the longitudinal glia in *sca-vnd* embryos typically assume a more lateral position [compare Fig. 7(A) and (C)]. The distribution of Repo is affected more strongly in embryos that over express *vnd* in the *Kr* domain or in *HS-vnd* embryos. In *Kr-vnd* embryos, expression of Repo in glial precursor cells is affected as early as stage 12 (data not shown). In stage 14 embryos, Repo expression is almost completely absent in the domain of the CNS where *vnd* over expression is targeted [Fig. 7(B,E)].

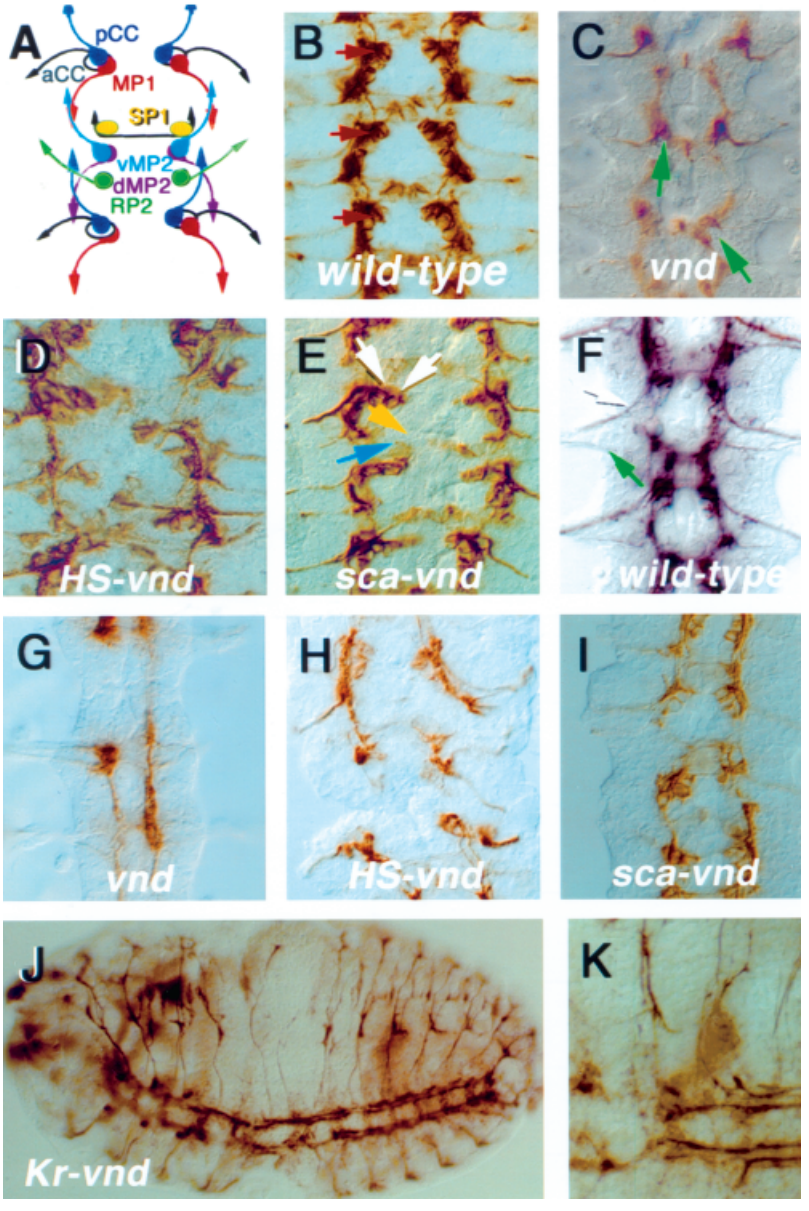

Figure 6

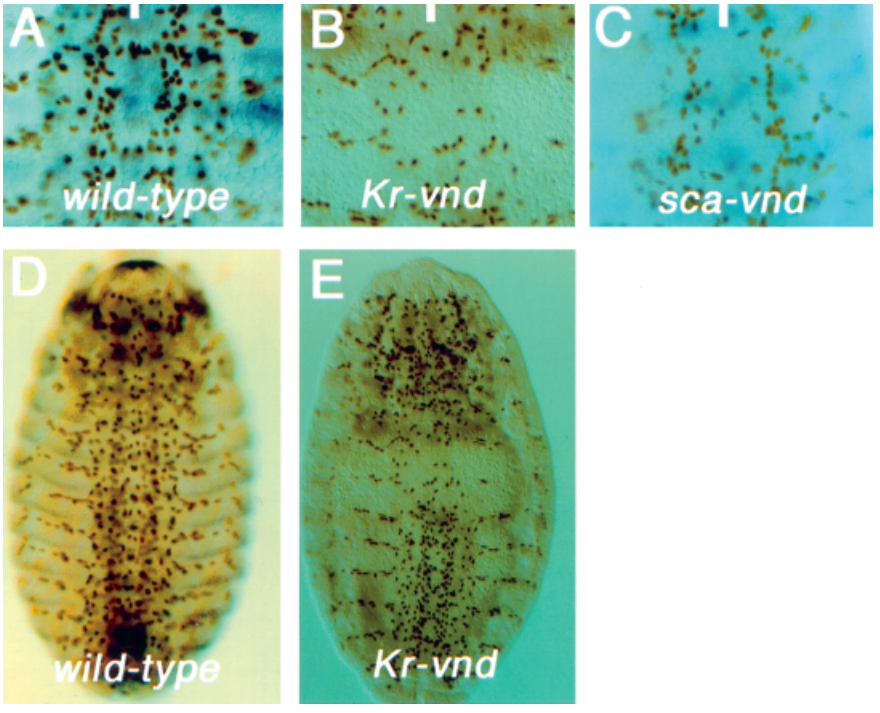

Figure 7 Longitudinal glial formation and/or positioning is affected by *vnd* over expression. Whole mount Repo staining of wild type [(A) and (D)], *Kr-vnd* [(B) and (E)], and *vnd-sca* (C) stage 14 embryos. Anterior is up. Three segments are shown in (A–C) [(A) and (D)]. The distribution of longitudinal glia in a wild-type embryo is shown. There are approximately six longitudinal glia per hemi-segment, organized in three rows. [(B) and (E)] In *Kr-vnd* embryos, the longitudinal glia are missing or are greatly reduced in number due to targeted *vnd* over expression in the *Kr* expression domain. (C) In *sca-vnd* embryos the number of longitudinal glia is reduced and they lie at an abnormal dorsal location compared to the wild-type pattern [see (A)].

Thus, Fas II staining of *vnd* embryos confirms that both the VUM and the RP2 are mis-specified in *vnd* mutants, and it reveals that *vnd* over expression mutants have an overall reduction in the number of Fas II expressed neurons. The aberrant distribution or absence of Repo in longitudinal glia of *vnd* over expression mutants explains the dorsal location of the longitudinal connectives.

Figure 8 is a schematic representation of the CNS phenotypes we have identified when *vnd* is missing or over expressed, while Table 2 summarizes the neurons and glia that are affected in *vnd* loss- and gain-of-function mutants. Clearly, disturbances in the normal *vnd* expression pattern dramatically affect CNS integrity.

DISCUSSION

The data presented here show that *vnd* levels must be tightly regulated for normal CNS specification. *vnd* mutation or over expression not only affects neuronal localization and number, as well as axonal pathfinding but also the specification of glia that are critical to CNS integrity. We show that lack of progeny of ventral neuroblasts caused by *vnd* mutation results in a collapsed CNS phenotype with weakened longitudinal connectives and fused commissures. Ectopic *vnd* expression results in longitudinal connectives that are aberrantly positioned too far from the ventral midline, due in part to the mis-specification of longitudinal glia that arise from the lateral neuroectoderm. In both *vnd* loss- and gain-of-function mutants the behavior and/or presence of commissural neurons are affected. Fas III-expressing commissural neurons are missing in *vnd* mutants, while *vnd* over expression results in commissural neurons that are generally missing or that project their axons away from the ventral midline. Neurons that arise from the ventral midline and an intermediate neuroblast are affected when *vnd* is missing in a noncell autonomous manner, whereas over expression of *vnd* results in the mis-

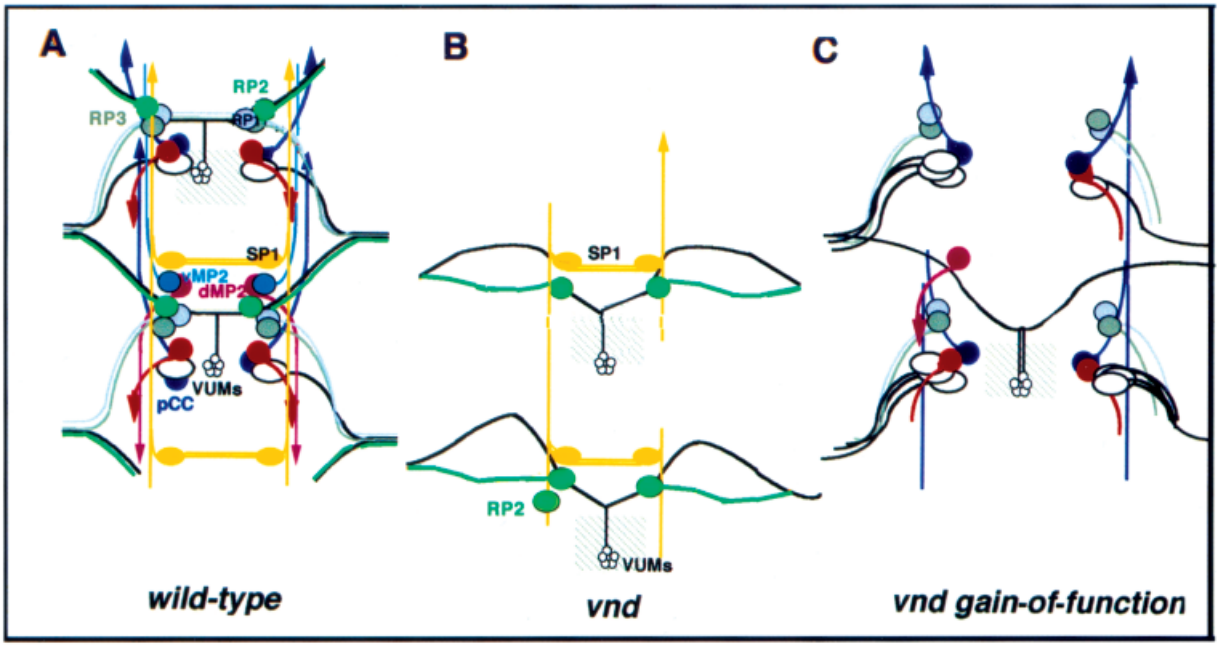

Figure 8 Summary of neuronal defects in *vnd* loss- and gain-of function mutants. Schematic of the organization, location, and axonal pathfinding of CNS neurons in stage 14 wild-type (A), *vnd* (B), and *vnd* over expression mutants (C) is presented. (A) In wild-type embryos, aCC (white), pCC (navy), and MP1 (red) are positioned in the anterior corners of the hemi-segment, while vMP2 (blue), dMP2 (pink), and SP1 (yellow) lie in the posterior corners of the hemi-segment. RP1 (light purple), RP2 (green), and RP3 (gray) sit posterior to these neurons. The VUM neurons (black) are also posteriorly located at the midline. The pCC, dMP2, RP2, and VUM cell bodies are located on the dorsal surface of the CNS; all other cell bodies are ventrally located. vMP2 and pCC pioneer a ventral ascending longitudinal connective, while MP1/dMP2 pioneer a descending dorsal connective. The posterolateral extending aCC and the anterolateral extending RP2/VUM fascicles pioneer the ISN. The RP1/RP3 fascicle pioneers the anterior commissure. [(B) and (C)] use the color scheme for neurons shown in (A). (B) In *vnd* embryos, aCC, pCC, MP1, vMP2, and dMP2 do not express 22C10, Fas II, or Eve (aCC/pCC). RP1 and RP3 fail to express Fas III and thus are either mis-specified or missing. RP2 is generally duplicated and/or aberrantly positioned. The ISN is aberrantly positioned because the VUM/RP2 fascicle is abnormal where the motor fascicles exit the CNS at an atypical position. The SP1 cell bodies are located too close to the ventral midline. (C) In embryos that ectopically express *vnd,* interneurons are abnormally positioned too far from the midline. Anterior hemi-segment neurons are over abundant, particularly aCC neurons, while posterior hemi-segment neurons express reduced amounts of 22C10 and Fas II.

specification of neurons and/or glia that arise from all dorsal-ventral levels of the developing CNS. Strikingly, midline glia are over represented and aberrantly located when *vnd* is over expressed, although *vnd* does not appear to regulate the expression of *Sim* directly. The data presented here indicate that progeny of the individual columnar stripes of *Drosophila* precursor cells do not develop as independent entities. Just as the lateral neuroectoderm is dependent on the mesectoderm for its specification (Menne et al., 1997; Lee et al., 1999; Chang et al., 2000), our data suggest that the reverse also holds true.

vnd Over Expression Results in the Mis-Specification of Both Neurons and Glia

Over expression of *vnd* leads to the mislocalization of interneurons too far from the ventral midline, abnormal pathfinding, and a reduction in the number of commissural neurons. *vnd* over expression also af-

Table 2 Summary of Neuronal and Glial Phenotypes Identified in *vnd* Loss- and Gain-of-Function Mutants Relative to Their Origin from Ventral (*vnd*+), Intermediate (*ind*+), Lateral (*msh*+), Neuroectoderm, and the Ventral Midline (*sim*+)

Neuron or Glia	Derives from	*vnd* Embryo	*vnd*+ to ++ Embryo
MP1*	Ventral midline *sim*+	Does not express 22C10	Variable phenotype
aCC†	Ventral neuroblast *vnd*+	−	Duplicated
pCC†	Ventral neuroblast *vnd*+	−	May be duplicated
Sp1*	?	+ but abnormal location near midline	−
vMP2*,‡	Ventral neuroblast *vnd*+	Does not express 22C10	−
dMP2‡	Ventral neuroblast *vnd*+	Does not express Fas II	−
VUMs	Midline neuroblast *sim*+	+, but have pathfinding defects	+
U and CQs	Ventral neuroblast *vnd*+	Do not express Eve	May be duplicated
RP1 and RP3	Ventral neuroblast *vnd*+	Do not express Fas III	Present pathfinding defects
RP2	Intermediate neuroblast *ind*+	+ sometimes, duplicated or abnormally positioned pathfinding defects	Generally missing
EL	Lateral neuroblast *msh*+	Does not express Eve	Affected in a dose-dependent manner
Midline glia	Midline *sim*+	Reduction in numbers late in development	Increase in number, aberrant positioning
Longitudinal glia	Lateral neuroectoderm *msh*+	+	Decrease in number, aberrant positioning

* MP1, SP1, and vMP2 were identified using 22C10 and Fas II antibodies.
† 22C10 selectively stains aCC but not pCC; but neurons express 22C10 and Eve.
‡ vMP2 and dMP2 express Fas II while Eve is selectively expressed in vMP2 but not dMP2.

fects both longitudinal and midline glia location and numbers. Midline glia are over produced, whereas the longitudinal glia numbers are reduced. In addition, both the midline and longitudinal glia are atypically located at a more dorsal location due to *vnd* over expression. The mislocalization of the midline glia potentially prevents them from performing their normal function in commissure formation and separation (Klambt et al., 1991).

Clearly the level of commissural crossing is reduced in embryos that over express *vnd.* Thus, the combined effects of midline glial mis-specification (the reduction in the number of longitudinal glia and their aberrant dorsal location, as well as the decrease in longitudinal connectives) must lead to an imbalance amongst the levels of repulsive and midline signals (for reviews see Jacobs, 2000; Rusch and Van Vactor, 2000; Giger and Kolodkin, 2001). The longitudinal glial defects caused by *vnd* over expression may contribute to the reduction in neuronal numbers as development proceeds, because glia are required for neuronal viability (Xiong and Montell, 1995; Booth et al., 2000), and vice versa (Kinrade et al., 2001).

Premature Cell Death and Pathfinding Anomalies Contribute to the *vnd* CNS Phenotype

vnd is required for the viability of neuronal progeny of ventral neuroblasts (Jimenez and Campos-Ortega, 1990). Hence, the activation of largely unidentified *vnd* target genes is essential for the generation of ventral neuroblast-derived neurons. *vnd* represses *ind* expression in ventral neuroblasts in wild-type embryos (McDonald et al., 1998). Thus, targets of *ind* are likely to be inappropriately expressed in ventral cells when *vnd*-dependent genes are not activated in *vnd* mutant embryos. As a result, the CNS is collapsed with only a fraction of the normal number of neurons. The aberrant specification and/or lack of RP1, RP3, and MP1, combined with abnormal VUM axonal pathfinding and a reduction in midline glial numbers accounts for the fused commissural phenotype in *vnd* mutants, because these cells are essential for commissure formation and separation (Klambt et al., 1991). Thus, lack of *vnd* in the lateral neuroectoderm also affects mesectodermal development. Disturbances in the normal *vnd* expression pattern also result in non-cell autonomous defects in the

RP2 neuron, which originates from the intermediate neuroblast 4.2, which does not express *vnd* (Broadus et al., 1995). The RP2 pathfinding defects in *vnd* mutants are potentially due to the absence of the aCC pioneer neuron, the RP2 target.

Vertebrate Orthologues of *vnd* Specify Neuronal Identity

How do the *vnd* loss- and gain-of-function phenotypes correlate with what is currently known about the homologues of *vnd* in vertebrate CNS specification? Many aspects of D-V patterning of the lateral neuroectoderm are highly conserved. The *Nkx 2.2, 2.1, 6.1, 6.2,* and *9.1* orthologues of *vnd* are expressed in parallel independent and overlapping domains in the ventral part of the neural tube. In addition, homologues of *ind* and *msh* are expressed in parallel domains in the vertebrate neural tube (for review and references see Cornell and Von Ohlen, 2000). There is a ventral to dorsal shift in the identity of neurons in *NKx2.2* and *2.1* knock-out mutants, as seen in *vnd* mutants. In *NKx2.2* mutants motor neurons are generated rather than interneurons (Briscoe et al., 1999), while in *NKx2.1* mutants the loss of pallidal structures and a subset of basal forebrain neurons results from a ventral to dorsal transformation of the pallidal primordium into a striatal-like anlage (Sussel et al., 1999). Recently, Briscoe et al. (2000) proposed that *Nkx2.2* activates the expression of the vertebrate homologue of *Sim, Sim-1,* based on an increased domain of *Sim* expression when *Nkx2.2* was over expressed. Although we see increased numbers of *Sim*-expressing cells in the late CNS of *Drosophila* embryos that over express *vnd,* prior to stage 12 the expression pattern of *Sim* was wild-type in both *vnd* loss- and gain-of-function mutants. This suggests that *vnd* does not directly regulate *Sim* expression in *Drosophila.*

How Do Transcription Factors Specify Cell and Lineage Identity?

The fact that regulated expression of critical transcription factors is essential within neuronal progenitors and in neurons themselves for their accurate specification is well documented (for recent reviews see Arendt and Nubler-Jung, 1999; Jurata et al., 2000). For example, in *Drosophila* the asymmetric localization of the transcription factor Prospero (Pros) in neuroblasts leads to its presence exclusively in the daughter GMCs. Pros nuclear expression leads to the exit of GMCs from the mitotically active state and their terminal differentiation (Hirata et al., 1995; Spana and Doe, 1995; Li and Vaessin, 2000). The transcription factor, Klumpfuss, is required in GMCs to distinguish between different sublineages (Yang et al., 1997), while Fushi tarazu and Eve expression in specific GMCs is essential for RP1, 2, and 3 axon guidance (Doe et al., 1988a,b). A host of transcription factors have now been implicated in axonal pathfinding, most notably the Lim-type homeodomain transcription factors, which specify motorneuron behavior using the "Lim code" (Lundgren et al., 1995; Thor and Thomas, 1997; Thor et al., 1999). However, the majority of the targets of transcription factors remain elusive at this time. Thus, at this time, it is not well understood how these proteins regulate the expression of critical downstream target genes to direct the specification of neurons so that they can correctly extend their axons to fasciculate appropriately.

Both D-V and A-P Patterning Genes Are Required for CNS Development

The data presented here indicate that *vnd* is an essential "columnar" D-V stripe gene responsible for both subdividing the neuroectoderm into stripes and specifying the lineage of individual neuroblasts. Clearly *vnd* expression must be tighly regulated for the specification of a variety of cell types within the developing CNS. Our data clearly support the hypothesis that pattern formation and cell-type specification are closely linked. The A-P patterning genes of the segment polarity group, including *wingless* and *gooseberry,* subdivide the neuroectoderm into rows. Like *vnd,* these genes specify not only the identity, but also the fate, of individual rows of neuroblasts (Chu-Lagraff and Doe, 1993; Skeath et al., 1995). The combined effects of both A-P and D-V patterning regulators not only make each neuroblast within a hemisegment unique; in addition, both these groups of patterning genes are essential to establish the finely tuned hierarchical network required so that individual neurons are appropriately specified and express the repertoire of essential proteins that makes each unique.

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