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 be tightly regulated.

Keywords: NK-2; mutant; neurons; glia; axons

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-
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 mis-specified, 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 pH5B/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%
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 wcDNA 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. 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).
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-
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.
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...
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 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.

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

The commisural 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 commisural 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 commisural 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 posteriolaterally, 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 neurophiltes. 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 normal 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 disrupted 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 posterolaterally 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/glia-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 overexpression. 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 overexpress 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 overexpression is targeted [Fig. 7(B,E)].
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-
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+)

<table>
<thead>
<tr>
<th>Neuron or Glia</th>
<th>Derives from</th>
<th>vnd Embryo</th>
<th>vnd+ to ++ Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP1*</td>
<td>Ventral midline sim+</td>
<td>Does not express 22C10</td>
<td>Variable phenotype</td>
</tr>
<tr>
<td>aCC1</td>
<td>Ventral neuroblast vnd+</td>
<td>—</td>
<td>Duplicated</td>
</tr>
<tr>
<td>pCC1</td>
<td>Ventral neuroblast vnd+</td>
<td>—</td>
<td>May be duplicated</td>
</tr>
<tr>
<td>Sp1*</td>
<td>?</td>
<td>+ but abnormal location near midline</td>
<td>—</td>
</tr>
<tr>
<td>vMP2*†</td>
<td>Ventral neuroblast vnd+</td>
<td>Does not express 22C10</td>
<td>—</td>
</tr>
<tr>
<td>dMP2‡</td>
<td>Ventral neuroblast vnd+</td>
<td>Does not express Fas II</td>
<td>—</td>
</tr>
<tr>
<td>VUMs</td>
<td>Midline neuroblast sim+</td>
<td>+, but have pathfinding defects</td>
<td>+</td>
</tr>
<tr>
<td>U and CQs</td>
<td>Ventral neuroblast vnd+</td>
<td>Do not express Eve</td>
<td>May be duplicated</td>
</tr>
<tr>
<td>RP1 and RP3</td>
<td>Ventral neuroblast vnd+</td>
<td>Do not express Fas III</td>
<td>Present pathfinding defects</td>
</tr>
<tr>
<td>EL</td>
<td>Intermediate neuroblast ind+</td>
<td>+ sometimes, duplicated or abnormally positioned pathfinding defects</td>
<td>Affected in a dose-dependent manner</td>
</tr>
<tr>
<td>Midline glia</td>
<td>Midline sim+</td>
<td>Reduction in numbers late in development</td>
<td>Increase in number, aberrant positioning</td>
</tr>
<tr>
<td>Longitudinal glia</td>
<td>Lateral neuroectoderm msh+</td>
<td>+</td>
<td>Decrease in number, aberrant positioning</td>
</tr>
</tbody>
</table>

* 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.

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 tightly 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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REFERENCES


Jimenez F, Campos-Ortega JA. 1990. Defective neuroblast...


