Development 133, 4331-4339 (2006) doi:10.1242/dev.02606

The columnar gene *vnd* is required for tritocerebral neuromere formation during embryonic brain development of *Drosophila*

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In *Drosophila*, evolutionarily conserved transcription factors are required for the specification of neural lineages along the anteroposterior and dorsoventral axes, such as Hox genes for anteroposterior and columnar genes for dorsoventral patterning. In this report, we analyse the role of the columnar patterning gene *ventral nervous system defective* (*vnd*) in embryonic brain development. Expression of *vnd* is observed in specific subsets of cells in all brain neuromeres. Loss-of-function analysis focussed on the tritocerebrum shows that inactivation of *vnd* results in regionalized axonal patterning defects, which are comparable with the brain phenotype caused by mutation of the Hox gene *labial* (*lab*). However, in contrast to *lab* activity in specifying tritocerebral neuronal identity, *vnd* is required for the formation and specification of tritocerebral neural lineages. Thus, in early *vnd* mutant embryos, the Tv1-Tv5 neuroblasts, which normally express *lab*, do not form. Later in embryogenesis, *vnd* mutants show an extensive loss of *lab*-expressing cells because of increased apoptotic activity, resulting in a gap-like brain phenotype that is characterized by an almost complete absence of the tritocerebral neuromere. Correspondingly, genetic block of apoptosis in *vnd* mutant embryos partially restores tritocerebral cells as well as axon tracts. Taken together, our results indicate that *vnd* is required for the genesis and proper identity specification of tritocerebral neural lineages during embryonic brain development of *Drosophila*.

KEY WORDS: *Drosophila*, Brain development, Neuromere, DV patterning, *ventral nervous system defective*, Hox gene, *labial*, Neuroblast, Programmed cell death

INTRODUCTION

In *Drosophila*, molecular genetic studies have identified genetic cascades that pattern and specify the embryonic ventral nerve cord (VNC) along the anteroposterior (AP) and dorsoventral (DV) axes. These studies show that segment polarity gene activity divides each segment along the AP axis into four parallel transverse rows, whereas columnar gene activity results in a tripartite dorsoventral subdivision of the neuroectoderm into longitudinal domains (Bhat, 1999; von Ohlen and Doe, 2000). Thus, *ventral nervous system defective* (*vnd*) is expressed in ventral neuroectodermal cells (Jimenez et al., 1995; Mellerick and Nirenberg, 1995), while *intermediate neuroblasts defective* (*ind*) expression is restricted to intermediate neuroectodermal cells (Weiss et al., 1998) and *muscle specific homeobox* (*msh*) is expressed in lateral neuroectodermal cells (D'Alessio and Frasch, 1996; Isshiki et al., 1997). These neuroectodermal cells in turn give rise to the three columns of ventral, intermediate and lateral neuroblasts. The different fates of the individual neuroblasts that form in different dorsoventral columns are influenced by expression of the corresponding columnar genes (reviewed by Skeath, 1999; Skeath and Thor, 2003).

The functional role of the columnar genes is exemplified by *vnd*, an *Nkx2*-type homeobox gene, which is continuously expressed within the developing VNC from cellularization until the completion of embryonic development. In *vnd* loss-of-function mutants, ventral neuroblasts are absent or mis-specified. These precursor cell defects correlate with loss or mis-specification of neuronal progeny, errors in axonal pathfinding and an overall reduced number of cells in the developing VNC of *vnd* mutant embryos. Thus, in *vnd* loss-of-function mutants, commissures are fused, ventral unpaired medial neurons show pathfinding defects and midline glia are reduced in number. Conversely, overexpression of *vnd* can lead to transformations in the identity of intermediate and lateral neuroblasts (Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002).

In contrast to the situation in the embryonic VNC, much less is known about the expression and function of the columnar gene *vnd* in the developing brain of *Drosophila*. A recent study on early brain neurogenesis shows that *vnd* is expressed in the procephalic neuroectoderm as well as in subsets of identified brain neuroblasts (Urbach and Technau, 2003a; Urbach and Technau, 2003b). However, information on the expression and function of *vnd* during later embryonic brain development is lacking.

Here, we analyse the role of the columnar patterning gene *vnd* during embryonic brain development of *Drosophila*. Using immunocytochemistry, we first map the expression of *vnd* and show that it is confined to subsets of neural cells in the developing protocerebrum, deuterocerebrum and tritocerebrum. We then carry out a functional analysis of *vnd* focussed on the intercalary segment and tritocerebral neuromeres, and show that *vnd* is essential for the formation of the tritocerebrum in the developing brain. Thus, loss of *vnd* function leads to a gap-like brain phenotype that is due to the defective formation of a subset of tritocerebral neuroblast and the

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Accepted 31 August 2006

subsequent loss of neural tissue in the mutant domain. Moreover, we show that this loss of neural tissue is associated with increased apoptotic activity, resulting in the loss of the tritocerebral commissure and the longitudinal connectives that normally run through this neuromere, whereas blocking apoptosis in *vnd*-null mutant embryos results in partial restoration of tritocerebral cells and axon tracts. These findings suggest that the dorsoventral patterning gene *vnd* is essential for development and identity specification of tritocerebral neural lineages in embryonic brain development of *Drosophila*.

MATERIALS AND METHODS

Drosophila strains and genetics

The wild type was Oregon-R. For *vnd* mutant analysis, the null allele *vnd*^{6} (Jimenez and Campos-Ortega, 1990) was used, balanced over FM7, ftz-*lacZ*. Homozygous null mutants were identified by the absence of *ftz-lacZ*. To analyse the development of the tritocerebral *lab* expression territory in the *vnd* mutant background, we used the line *7.31 lab-lacZ/7.31 lab-lacZ* (Tremml and Bienz, 1992) crossed into *vnd*^{6}. To identify *vnd* expression in former *lab*-expressing tritocerebral cells in the *labial* mutant background, we used line *7.31 lab-lacZ/7.31 lab-lacZ; lab*^{vd1}*/TM3, hb-lacZ* (Tremml and Bienz, 1992). Homozygous null mutants were identified by the absence of *hb-lacZ*. For comparison with the wild-type situation, *7.31 lab-lacZ* was crossed back to wild type. *7.31 lab-lacZ* shows cytoplasmic distribution of *βgal* and reflects endogenous *lab* expression with additional ectopic expression patterns in the deutocerebral anlage (Hirth et al., 1998; Hirth et al., 2001). The UAS/Gal4 transcriptional activation system (Brand and Perrimon, 1993) was used in order to perform overexpression experiments. As a Gal4-driver line, we used the *sca::*Gal4 (Klaes et al., 1994; Sprecher et al., 2004) which in the embryonic CNS is active from neurectodermal stage to early neural lineage formation. For the ubiquitous block of apoptosis within neural lineages of *vnd*^{6} mutant embryos, we used the *sca::*Gal4 driver line in order to activate UAS::*p35* transcription (Mergliano and Minden, 2003). Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

Immunocytochemistry and TUNEL assay

Embryos were dechorionated, fixed, immunostained, flattened and staged according to previously published protocols (Patel, 1994; Therianos et al., 1995; Urbach et al., 2003). Primary antibodies were rabbit anti-Deadpan [1:300 (Bier et al., 1992); kindly provided by H. Vaessin], rabbit anti-HRP [FITC-conjugated; 1:100 (Jan and Jan, 1982); Jackson Immunoresearch], rabbit anti-LAB at 1:100 (F.H., unpublished), rat anti-LAB at 1:500 (F.H., unpublished), mouse anti-NRT at 1:20 (BP106 antibody, DSHB), rabbit anti-VND at 1:200 (McDonald et al., 1998) (kindly provided by C. Q. Doe), rabbit anti-βGAL 1:200-1:400 (Milan Analytika), mouse anti-βGAL 1:50 (DSHB), mouse anti-Fasciclin II 1:5 (Lin and Goodman, 1994), rat anti-ELAV 1:30 (DSHB), mouse anti-PROS 1:4 (Spana and Doe, 1995), mouse anti-REPO 1:20 (DSHB), mouse anti-Engrailed [4D9,1:6 (Patel et al., 1989); DSHB]. Secondary antibodies used for confocal microscopic analysis were Alexa-488, Alexa-568 and Alexa-647 antibodies generated in goat (Molecular Probes), all at 1:150 dilution. Secondary antibodies used for flat-mount preparations analyzed using Nomarski optics were either biotinylated or alkaline phosphatase-conjugated antibodies generated in goat all at 1:500 (Dianova). Apoptotic activity was assayed by TUNEL analysis using a commercial TUNEL kit (ApoTag, Oncor) as previously described (Richter et al., 1998) with the following modifications. After fixation, embryos were washed in PBT for 2×5 minutes, then washed in Equilibration Buffer (from the ApoTaq kit) for 2 minutes. Embryos were incubated in the working strength TdT mixture (from the ApoTaq kit) for 1 hour at 37°C. After incubation, supernatant was removed and embryos were washed for 2×2 minutes with Stop/Wash solution (from the ApoTaq kit), and subsequently washed for 3×2 minutes, 2×30 minutes in PBT before starting immunolabelling. Embryos were mounted in Vectashield H-1000 (Vector).

Laser confocal microscopy and generation of 3D digital models

For laser confocal microscopy, a Leica TCS SP was used. Optical sections ranged from 0.2-1.5 μm recorded in line average mode with picture size of 512×512 pixels, or 1024×1024 pixels. Captured images from optical sections were arranged and processed using IMARIS (Bitplane). Complete series of optical sections were imported and processed using ImageJ. For the generation of 3D digital models, raw tiff stacks (stacks of optical sections) were imported into AMIRA (Mercury Computer Systems). User-defined materials were drawn manually around the labelled structures (immunoreactivity of a given antibody) in each layer of a given tiff stack, which were to be included in the model. Subsequently, the program synthesizes a surface by triangulation around the defined materials, which was further processed by increasing the number of triangles per material and smoothening of the surface. For 2D representations in figures, screen shots were generated showing the model in the appropriate angle, virtual lighting and transparency of individual material surfaces (for details, see Pereanu and Hartenstein, 2004). Figures were arranged and labelled using Adobe Photoshop.

RESULTS

Neuromere-specific *vnd* expression during embryonic brain development

During an initial phase of embryonic neurogenesis, *vnd* expression is seen in the neurectoderm and delaminating neuroblasts in the ventral domains of the protocerebral, deutcerebral and tritocerebral brain neuromeres [for a detailed description of *vnd* expression during the early phase of neurogenesis, Urbach et al. (Urbach et al., 2006)]. During later stages of embryogenesis, *vnd* expression is also seen in specific cell clusters within these brain neuromeres. Thus, at late stage 12, a large expression domain is seen in the protocerebral neuromere (Fig. 1A,B) and two smaller expression domains are observed in the deutocerebrum and in the tritocerebrum. Although the tritocerebral and deuterocerebral *vnd* expression clusters are in

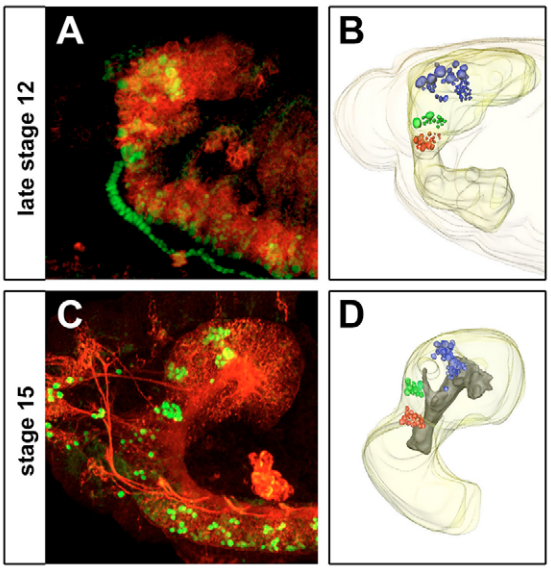

Fig. 1. Spatiotemporal expression of *vnd* during embryonic brain development. Laser confocal microscopy, reconstructions of optical sections, lateral views (A,C) and 3D reconstructed models of confocal microscopic stacks, covering corresponding optical sections (B,D) are shown for embryonic late stage 12 (**A**,**B**), and stage 15 (**C**,**D**). (A) Embryo double-immunolabelled with anti-Neurotactin (NRT) antibody (red) and anti-VND antibody (green/yellow). (C) Double-immunolabelling with anti-HRP antibody (red) and anti-VND antibody (green, yellow). (A,C) At stage late 12, three *vnd* expression domains become apparent that are still observable at stage 15. (B,D) 3D reconstructed models show relative location of domains within developing brain (*vnd* expression domains: blue, protocerebral; green, deuterocerebral; red, tritocerebral).

close proximity to each other, they do not overlap. Towards the end of embryogenesis, at embryonic stage 15, expression of *vnd* is still visible in these three neuromeric domains (Fig. 1C,D). Throughout embryonic neurogenesis, we find *vnd* expression in neuroblasts, ganglion mother cells and neurons as judged by immunolabelling with anti-PROS and neuron-specific anti-ELAV antibodies. Immunolabelling with glia-specific anti-REPO antibody indicates that none of the glia cells of the embryonic brain express *vnd* (data not shown). *vnd*-expressing cells are also seen in the neuromeres of the suboesophageal ganglion and the VNC, as well as in peripheral sense organs; these *vnd*-expressing cells will not be considered further in this report.

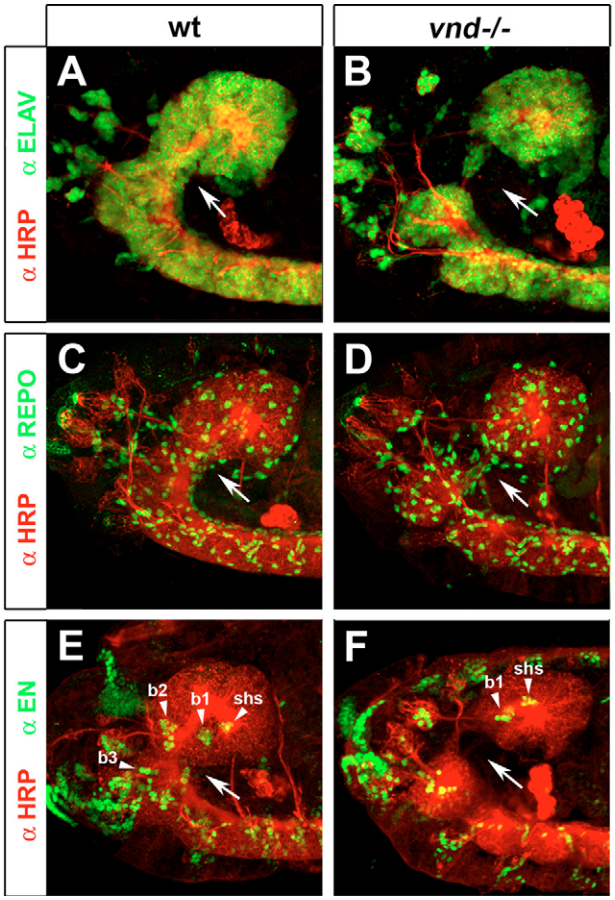

Fig. 2. Mutant brain phenotype observed in *vnd*-null mutant embryos at embryonic stage 15. Laser confocal microscopy reconstructions of optical sections, lateral views. (**A**,**B**) Double-immunolabelling with anti-HRP antibody (red) and anti-ELAV antibody (yellow/green). (**C**,**D**) Double-immunolabelling with anti-HRP antibody (red) and glial-specific anti-REPO antibody (yellow/green). Arrows indicate general trito-deutocerebral region. (**E**,**F**) Double immunolabelling with anti-HRP antibody (red) and an anti-EN antibody (yellow/green). (A) In wild type, neuron-specific marker ELAV reveals all neural cell bodies. (B) By contrast, in *vnd*-null mutants a large gap is seen in the tritocerebral/deuterocerebral region (arrow). (C) The glia-specific marker REPO reveals localization of glial cell bodies in embryonic wild-type brain. (D) In *vnd* mutant embryos, REPO-expressing cells in residual tritocerebral/deuterocerebral region appear to be present but are severely misplaced (arrow). (E) In wild type, the protocerebral b1 *en*-stripe (b1), deuterocerebral b2 *en*-stripe (b2), tritocerebral b3 *en*-stripe and anteriormost *en* expressing secondary head spot (shs) are visible (arrowheads). (F) By contrast, in *vnd*-null mutant embryos only b1 *en*-stripe and *en* expressing secondary head spot are present (arrowheads), and neuron-specific HRP marker reveals a cellular gap in deuto- and tritocerebral region (arrow).

Gap-like brain defects occur in *vnd* loss-of-function mutants

Mutational inactivation of *vnd* results in a pronounced brain phenotype in the late stage embryonic brain. Immunolabelling with neuron-specific anti-HRP and anti-ELAV antibodies identifies a large gap separating the anterior deutocerebral brain region from the neuromeres of the posterior suboesophageal ganglion (Fig. 2A,B). Evaluation of the penetrance of the *vnd*-null mutant phenotype reveals that in 36.5% of the mutant embryos (27/74) this gap is completely devoid of Elav- and HRP-immunoreactive cells (not shown), while in the majority of *vnd* mutant embryos (63%; 46/74) a thin strand of ELAV- and HRP-immunoreactive cells remains and interconnects the protocerebrum and the suboesophageal ganglion (Fig. 2B). This cell loss is associated with axonal patterning defects in the embryonic brain. The longitudinal connectives that normally run from the protocerebrum to the suboesophageal ganglion are missing or strongly reduced (Fig. 2, arrows) and the tritocerebral commissure is completely absent. Glia-specific anti-REPO immunoreactivity reveals that glial cells are present in the mutant but fail to be correctly localized in the affected region, most probably owing to the absence of neuronal tissue (Fig. 2C,D).

To delineate the region affected in *vnd* mutants in more detail, we studied the expression of *engrailed* (*en*), which in the wild-type embryonic brain is located in several small clusters of cells that demarcate the posterior boundary of the brain neuromeres (Schmidt-Ott and Technau, 1992; Hirth et al., 1995). The b1 *en*-stripe (or *en* head spot) delimits the posterior protocerebrum (several *en* cells are also seen more anteriorly in the protocerebrum as the secondary head spot), the b2 *en*-stripe (or *en* antennal stripe) delimits the posterior deutocerebrum, and the b3 *en*-stripe (or *en* intercalary stripe) delimits the posterior tritocerebrum (Fig. 2E). In late *vnd* mutant brain (embryonic stage 13 onwards), only the b1 *en*-stripe and the secondary head spot are visible; neither the b2 *en*-stripe nor the b3 *en*-stripe can be identified (Fig. 2F). This supports the observation that major parts of the embryonic tritocerebrum and parts of the deutocerebrum are lacking in the *vnd* mutant. In addition to the cell loss defect in the tritocerebral/deutocerebral brain region, a less marked reduction in overall size of the protocerebrum is also seen in *vnd* mutant embryos. Moreover the organization of the suboesophageal ganglion and the VNC is affected in the *vnd* mutant (see also Mellerick and Modica, 2001). These latter two phenomena were not studied further.

At the gross histological level, the *vnd* mutant brain phenotype described above is, in part, reminiscent of the mutant brain phenotype observed for the Hox gene *labial* (*lab*). In *lab*-null mutants, tritocerebral cells are generated and positioned correctly; however, these cells fail to differentiate into neurons and marked axogenesis defects occur, including the disruption of longitudinal connectives and lack of the tritocerebral commissure (Hirth et al., 1998; Page, 2000; Hirth et al., 2001). As *lab* and *vnd* also show overlapping expression in a subset of tritocerebral neuroblasts (Urbach and Technau, 2003a), these findings suggest that *lab*-expressing tritocerebral neuroblasts are affected in *vnd* mutant embryos. To investigate this, we focussed on the developing tritocerebrum, and specifically on the *lab* expression domain of this neuromere, and first determined whether loss of *vnd* function affects formation of *lab*-expressing neuroblasts.

Defective tritocerebral neuroblast formation in *vnd* mutants

During the early phase of brain neurogenesis, the *lab*-expressing neuroectodermal domain gives rise to 15 neuroblasts, which include all of the tritocerebral neuroblasts and two deutocerebral neuroblasts (Urbach and Technau, 2003a). By stage 11, all of these neuroblasts are present and express *lab*; they include a ventral group of tritocerebral neuroblasts, Tv1-Tv5, a more dorsal group of tritocerebral neuroblasts, Td1-Td8, and two deutocerebral neuroblasts, Dv2 and Dv4 (Fig. 3A,B). In the wild type, the most ventral part of the neuroectodermal domain, from which the tritocerebral neuroblasts Tv1-Tv5 and the two deutocerebral neuroblasts originate, dynamically co-expresses *lab* and *vnd* between stages 8 and 11 (Urbach and Technau, 2003a).

In *vnd* mutants this ventral-most part of the *lab*-expressing domain appears to be reduced in size and accordingly, the number of *lab*-expressing neuroblasts that derive from this brain region is diminished (Fig. 3C,D). Generally only four to six large rounded cells are observed that co-express *lab* and the neuroblast-specific marker Deadpan (this may be a slight underestimate as a few enlarged rounded cells in sub-ectodermal position lacking Deadpan expression are sometimes observed in this region). Based on the expression of molecular markers indicative of dorsal neuroblasts [e.g. *ladybird early*, *empty spiracles*, *wingless* (Urbach and Technau, 2003a; Urbach et al., 2006)], this reduction in *lab*-expressing neuroblasts appears to affect preferentially ventral neuroblasts of the tritocerebrum and adjacent part of the deutocerebrum. These data imply that *vnd* is required for the formation of a ventral subset of *lab*-expressing neuroblasts in the developing tritocerebrum.

Although the reduction in tritocerebral neuroblast number seen in *vnd* mutants can account for some of the tritocerebral defects, this mechanism alone is unlikely to be the exclusive cause for the massive cell loss phenotype observed in the late embryonic *vnd* mutant brain. This is because a dorsal subset of the *lab*-expressing tritocerebral neuroblasts, as well as large number of *lab*-expressing neural progeny are generated in the tritocerebrum of stage 11 *vnd* mutant brains (compare Fig. 3B with 3D). Hence, in addition to defective neuroblast formation, other phenomena must be responsible for the gap-like phenotype observed in *vnd* mutant brains, implying that *vnd* is required also later in embryogenesis – either by acting directly on *lab* expression in tritocerebral cells or through a *lab*-independent requirement.

vnd and *lab* in tritocerebral neuromere formation

To investigate this, we first determined whether *vnd* and *lab* show overlapping expression during later stages of tritocerebral neuromere formation. Immunocytochemical analysis indicates that a partial overlap of *vnd* and *lab* expression persists in the differentiating tritocerebrum throughout embryogenesis and is prominent in the ventral region (according to neuraxis) of this neuromere (Fig. 4A,B). Next, we analysed *lab* expression in late *vnd* loss-of-function mutant brains. Owing to extensive cell loss in the *vnd* mutant tritocerebrum, this analysis was limited to the remaining strand of cells that interconnects the protocerebrum and the remaining part of the deutocerebrum with the suboesophageal ganglion. Despite the extensive cell loss seen in *vnd* mutant brains, remaining cells of the interconnecting strand do show *lab* expression (Fig. 4C,D).

We next investigated whether expression of *vnd* occurs in the *lab* mutant tritocerebrum by studying *lab* loss-of-function mutants. For this, we took advantage of the fact that in *lab*-null mutants, cells in the tritocerebral mutant domain are generated and can be visualized by a 7.31 *lab-lacZ* reporter construct (Tremml and Bienz, 1992; Hirth et al., 1998). Surprisingly, despite the lack of expression of neuronal differentiation markers in cells of the *lab* mutant domain (see also Hirth et al., 1998), *vnd* is expressed normally and shows partial overlap with tritocerebral *lab* mutant cells, as visualized by the *lab*-specific reporter construct (Fig. 4F). This indicates that expression of *vnd* is not affected by the absence of *lab* during late stages of embryonic brain development.

Increased apoptosis in the *vnd* mutant tritocerebrum

What is the nature of the *vnd* requirement for proper tritocerebral neuromere development during later stages of embryogenesis? One possibility is that *vnd* might be involved in maintaining differentiating cells during tritocerebral neuromere formation. To investigate this, we focussed on the tritocerebral cells that are labelled by the 7.31 *lab-lacZ* reporter construct and compared wild-

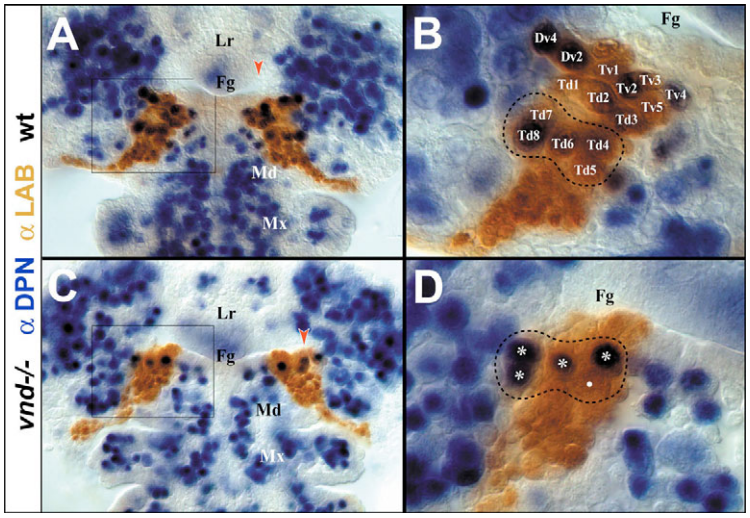

Fig. 3. Defective neuroblast formation in *lab*-expressing tritocerebral domain of *vnd* mutants. (**A-D**) Double-immunolabelling with neuroblast-specific anti-DPN (blue) and anti-LAB (brown), at embryonic stage 11, in wild type (WT) (A,B) and *vnd*-null mutants (C,D). (A-D) Ventral views of flat preparations. (B,D) Higher magnification of regions indicated in A,C by black frames, at level of brain neuroblasts. (A) In wild-type embryos, all brain neuroblasts have developed by stage 11. (B) Two deutocerebral and complete set of tritocerebral neuroblasts developing from LAB domain are indicated [according to nomenclature of Urbach et al. (Urbach et al., 2003)]: Tv1-5, ventral tritocerebral neuroblasts; Td1-8, dorsal tritocerebral neuroblasts; Dv2, Dv4 ventral deutocerebral neuroblasts. Broken line encircles group of dorsal neuroblasts that are assumed to be retained in *vnd*-null mutants (compare with D). (C) In *vnd*-null mutants, overall expansion of LAB domain appears to be reduced when compared with wild type (A), and invagination of the foregut (Fg) is affected (compare lateral extension of foregut invagination as marked by red arrowheads in A and C). (D) Number of DPN-positive neuroblasts (white asterisks) is diminished, when compared with B. One neuroblast (white dot) does not express DPN at detectable levels; similarly, in wild type a neuroblast expressing DPN at significantly lower levels is found in same relative position (see Td5 in cluster of neuroblasts encircled by broken line, B). Fg, foregut; Lr, labrum; Md and Mx, mandibular and maxillary segment, respectively.

type with *vnd* null mutant embryonic brains. At stage 12, expression of this reporter construct in a wild-type background reflects endogenous *lab* expression in the tritocerebrum (Fig. 5A,B,D,E). A comparison of reporter gene expression in wild type (Fig. 5B) with reporter gene expression in *vnd*-null mutants (Fig. 5C) at early stage 12 indicates that the *lab*-expressing tritocerebral domain is somewhat reduced in size but not deleted in *vnd* mutants (see also Fig. 3B,D). By contrast, by late stage 12, the *lab*-expressing tritocerebral domain is significantly reduced in *vnd* mutants (Fig. 5F). Thus, an average of 27 cells expressing the *lab-lacZ* reporter gene are detectable in the *vnd* mutant tritocerebrum when compared with an average of 43 cells detectable in wild type (Fig. 5K). This suggests that in addition to defective neuroblast formation, loss of *vnd* function affects proper development of neural cells in the tritocerebral *lab*-expression domain.

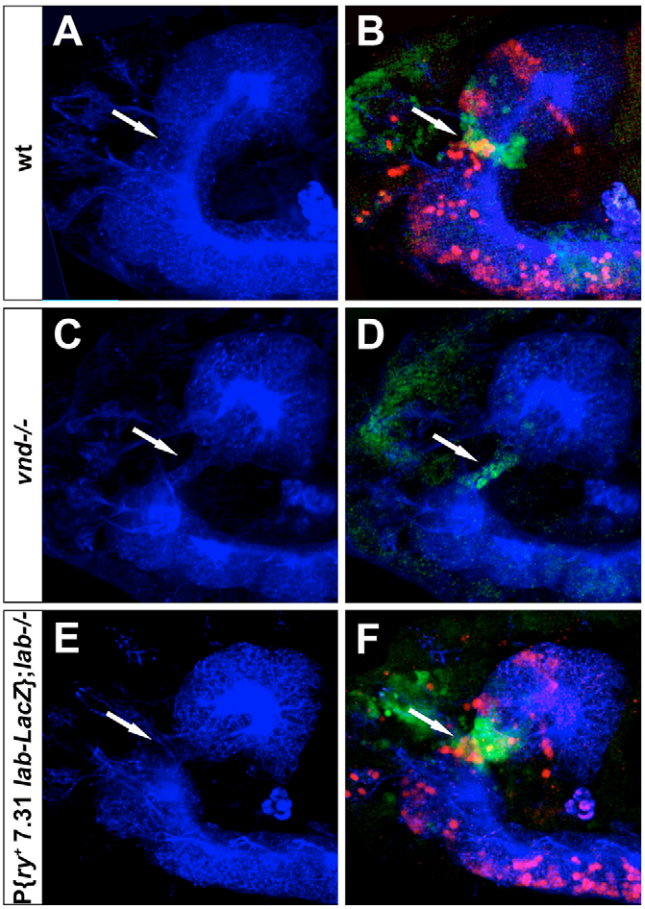

Fig. 4. *vnd* and the anterior Hox gene *labial* act independently in tritocerebral neuromere formation. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections, lateral views. Arrows indicate tritocerebral region. (**A**) Wild-type embryonic brain immunolabelled with anti-HRP antibody (blue). (**B**) Wild-type embryonic brain triple-immunolabelled with anti-HRP (blue), anti-LAB (green) and anti-VND (red); co-expression of *vnd* and *lab* is seen in part of *lab*-expressing tritocerebral domain (arrow); A and B are from same section. (**C**) *vnd* mutant embryonic brain immunolabelled with anti-HRP (blue). (**D**) *vnd* mutant embryonic brain double-immunolabelled anti-HRP (blue) and anti-LAB (green); only a few cells remain in the tritocerebrum and express *lab*; C and D are from same section. (**E**) *P{ry$^+$ 7.31 lab-LacZ};;lab^{vd1}/ lab^{vd1}*: null mutant embryonic brain immunolabelled with anti-HRP (blue); no anti-HRP immunoreactivity is detected in tritocerebral domain. (**F**) *P{ry$^+$ 7.31 lab-LacZ};;lab^{vd1}/ lab^{vd1}*: null mutant embryonic brain triple-immunolabelled with anti-HRP (blue), anti-VND (red) and anti-βGAL, revealing 7.31 *lab-LacZ* reporter (green). *vnd* expression is seen in a part of tritocerebral domain mutant for *lab* and expression overlaps with *lab-lacZ* specific reporter gene expression; E and F are from same section.

Analysis of programmed cell death reveals that this loss of neural cells in the *lab*-expressing domain is associated with increased apoptotic activity in the developing tritocerebrum. In early stage 12 wild type, low level apoptotic activity is associated with the tritocerebral *lab*-expressing domain as assayed by TUNEL staining (Fig. 5G). [A low level of apoptosis is seen throughout the developing brain at this stage (Abrams et al., 1993; Nassif et al., 1998).] In early stage 12 *vnd* mutants, a significant increase in the number of TUNEL-positive apoptotic cells is seen associated with the tritocerebral *lab*-expressing domain when compared with wild type and this correlates with a decrease in the number of *lab*-expressing cells in this region (Fig. 5H). Accordingly, by late stage 12, a significant reduction of the number of anti-LAB positive cells has occurred in *vnd* mutants when compared with wild type (Fig. 5I,J). Thus, on average, 20 TUNEL-positive apoptotic cells are identifiable within the *vnd* mutant tritocerebral *lab*-expression domain when compared with 10 apoptotic cells in wild type (Fig. 5K). These findings suggest that marked reduction of tritocerebral *lab*-expressing cells occurs as early as embryonic stage 12 and that this reduction is caused by increased apoptosis of neural tissue.

In order to further substantiate these observations, we next determined whether blocking apoptosis can prevent cell loss in the *vnd* mutant brain. For this, we used the UAS/Gal4 transcriptional activation system (Brand and Perrimon, 1993) in order to perform misexpression experiments in *vnd* mutant embryos. For the ubiquitous block of apoptosis from neurectodermal stage to early neural lineage formation, we used the *sca::*Gal4 driver line (Sprecher et al., 2004) in order to activate UAS::*p35* transcription, an inhibitor of cell-death effector caspases (Mergliano and Minden, 2003), in a *vnd*-null mutant background. When compared with wild type (Fig. 6A) and *vnd* mutant brain (Fig. 6B), ubiquitous block of apoptosis leads to a remarkable restoration of HRP-immunoreactive tissue in the *vnd* mutant tritocerebrum (Fig. 6C). Notably, and in contrast to the *vnd* mutant situation, descending longitudinal connectives that transverse the tritocerebrum are detectable (Fig. 6C, arrow). Moreover, a pronounced *lab*-expression domain is observed in the cell death prevented *vnd* mutant tritocerebrum (compare Fig. 6D with Fig. 4B). These data suggest that *lab* and *vnd* act in a genetically independent manner in tritocerebral neuromere development: the expression of *lab* appears to be largely unaffected by the absence of *vnd* as long as apoptosis is prevented.

To characterize in more detail the extent of neural tissue restoration of the *vnd* mutant brain phenotype resulting from block of apoptosis, we carried out immunocytochemistry using antibodies against Fasciclin II (FAS2) (Lin and Goodman, 1994) and ELAV. In the wild-type embryonic brain, anti-FAS2 immunostaining labels a number of early differentiating neurons, as well as axon tracts (Fig. 7A), whereas anti-ELAV labels differentiating postmitotic neurons (Fig. 7B). In the *vnd* mutant tritocerebrum, both FAS2 and ELAV-positive cells, as well as FAS2-immunoreactive longitudinal axon tracts, are severely perturbed or lacking (Fig. 7C,D; arrow). When compared with wild-type and *vnd* mutant brain, ubiquitous block of apoptosis significantly reduces the gap-like defects observed in *vnd* loss-of-function mutants. Thus, FAS2-immunoreactive longitudinal connectives are detectable and form a continuous band along the anteroposterior neuraxis. However, neural fibres contributing to longitudinal axon tracts display fasciculation defects and appear

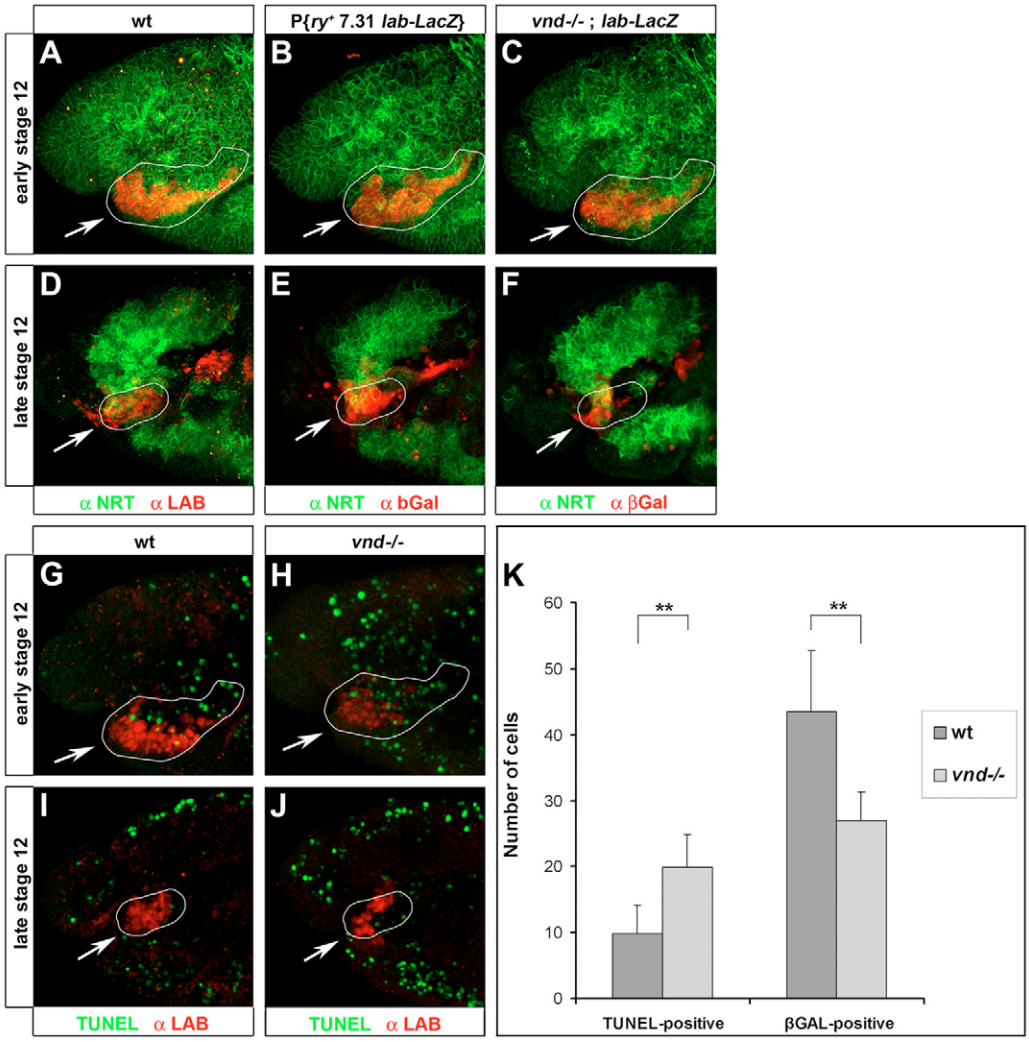

Fig. 5. Increased apoptosis in *vnd*-null mutant embryos at embryonic stage 12. Laser confocal microscopy, reconstructions of optical sections, lateral views. (**A-C**,**G**,**H**) Embryos at early stage 12; average maximal extent of *lab*-expressing domain in wild-type embryos is outlined (white line, arrow) and projected onto each figure in top row. (**D-F**,**I**,**J**) Embryos at late stage 12; average maximal extent of *lab*-expressing domain in wild-type embryos is outlined (white line, arrow) and projected onto each figure in bottom row. (A,D) Wild type double-immunolabelled with anti-NRT (green) and anti-LAB (red) showing *lab*-expression domain (arrow). (B,E) P{ry$^+$ 7.31 *lab*-LacZ} in wild-type background. Double-immunolabelling using anti-NRT (green) and anti-βGAL shows that 7.31 *lab-LacZ* reporter construct mimics endogenous *lab* expression. (C,F) P{ry$^+$ 7.31 *lab*-lacZ} in *vnd*-null background. Double-immunolabelling using anti-NRT (green) and anti-βGAL reveals extent of *lab* expression domain, as assayed by 7.31 *lab-lacZ* reporter construct. (G,I) Wild-type double-immunolabelled with anti-LAB (red) and TUNEL staining (green) showing low level of apoptotic activity in *lab* domain. (H,J) *vnd*-null mutant; anti-LAB immunolabelling (red) and TUNEL staining (green) showing increased level of apoptotic activity in *lab* expression domain at early stage 12. (**K**) Quantitation of TUNEL-positive cells and of β-gal-positive 7.31 *lab-lacZ*-expressing cells detectable in wild-type and *vnd* mutant background. Values are means of *n*=17 preparations counted in each case: wt TUNEL=10, *vnd* TUNEL=20; wt cells=43, *vnd* cells=27. Standard deviations are indicated as bars (P<0.000005 each) of Student's *t*-test are indicated as stars (**). By early stage 12, the number of TUNEL-positive apoptotic cells in *vnd* mutant tritocerebrum are significantly increased; by late stage 12, number of *lab-lacZ* expressing cells in *vnd* mutant tritocerebrum are significantly reduced.

only loosely bundled when compared with wild type (Fig. 7E, arrow). Moreover, a large number of ELAV-positive cells are detectable in the cell death prevented *vnd* mutant tritocerebrum (Fig. 7F, arrow). These data provide further evidence that the gap-like brain defect observed in *vnd* mutants is largely due to increased apoptotic activity and the subsequent loss of neural cells in the tritocerebral domain.

DISCUSSION

Expression and function of *vnd* in embryonic brain development

Previous analyses have demonstrated that the genes *vnd*, *ind* and *msh* are required for the columnar subdivision of the VNC neuroectoderm and subsequent formation and determination of neuroblasts along the dorsoventral axis during *Drosophila* embryogenesis (reviewed by Skeath and Thor, 2003). In the case of *vnd*, detailed studies have shown that *vnd* is required for specification of the ventral neuroectodermal column and specific neuroblasts, and that absence or misspecification of ventral neuroblasts correlates with the loss or mis-specification of neural progeny. For example, the aCC/pCC and dMP2/vMP2 neurons are lost, and the RP2 neuron is frequently absent in *vnd* mutant embryos, resulting in axonal pathfinding defects and defective commissure formation in the developing VNC (Jimenez et al., 1995; Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002).

In this study, we show that the *vnd* gene is also required during embryonic brain development. Starting at early stages of neuroectoderm and brain neuroblast formation, *vnd* expression is seen in the developing neuromeres of the embryonic brain (Urbach and Technau, 2003b; Urbach et al., 2006) and in the subsequent course of embryogenesis, *vnd* expression is seen in specific clusters of differentiating neural cells in each brain neuromere. For an investigation of the functional role of *vnd* in brain development, we focused on the tritocerebral neuromeres, where our mutant analysis suggests that *vnd* acts at least during two important steps in its development: precursor cell development and neural progeny

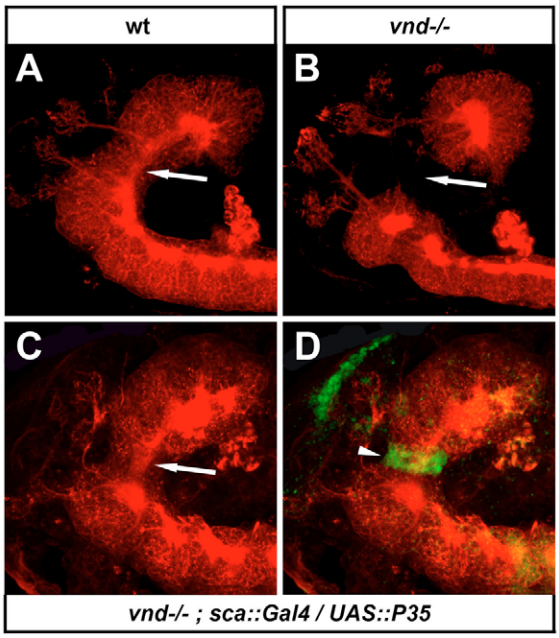

Fig. 6. Partial restoration of brain structures and *lab* expression in *vnd* mutants by blocking apoptosis. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections, lateral views. Arrows indicate tritocerebral region. (**A**) Wild type; (**B**) *vnd* mutant; (**C**,**D**) *sca*::Gal4/UAS::*p35* in *vnd* null mutant background. (A-C) Embryonic brain immunolabelled with anti-HRP (red). (D) Embryonic brain double-immunolabelled with anti-HRP (red) and anti-LAB (green). When compared with wild-type (A) and *vnd* mutant brain (B), *p35*-mediated block of apoptosis partially restores HRP-immunoreactive tissue in *vnd* mutant tritocerebrum, and descending longitudinal connectives that transverse the tritocerebrum are detectable (C). Restoration of neural tissue also results in wild-type-like *lab* expression domain in *vnd* mutant tritocerebrum (D, compare with Fig. 4B).

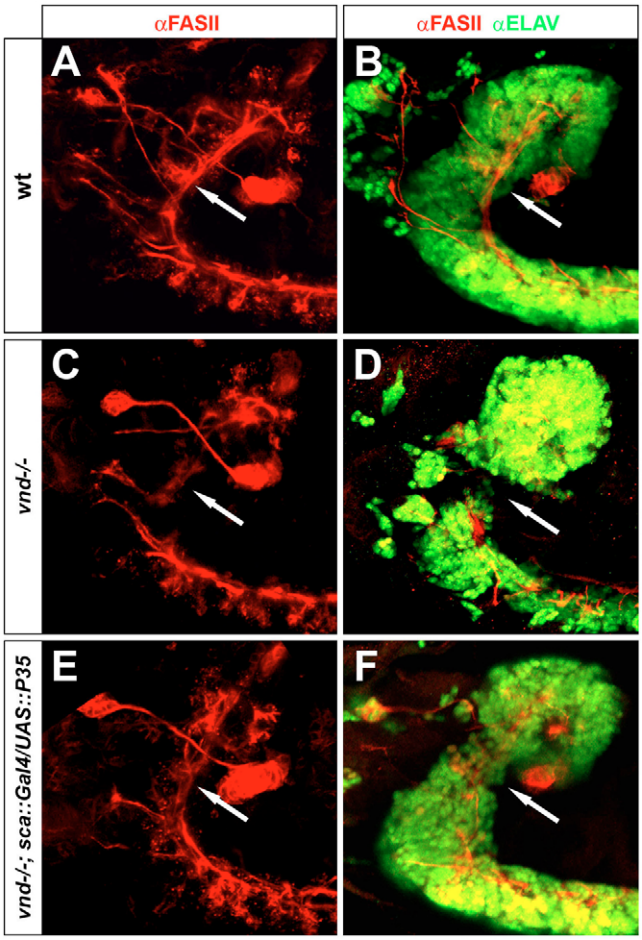

Fig. 7. Partial restoration of brain tracts in cell death prevented *vnd* mutant. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections, lateral views. Arrows indicate tritocerebral region. (**A**,**B**) Wild-type; (**C**,**D**) *vnd* mutant; (**E**,**F**) *sca*::Gal4/UAS::*p35* in *vnd*-null mutant background. Embryos in B,D,F are not the same as in A,C,E, respectively. (A,C,E) Embryonic brain immunolabelled with anti-FAS2 (red). (B,D,F) Embryonic brain double-immunolabelled with anti-FAS2 (red) and anti-ELAV (green). (A,B) In wild type, FAS2 immunostaining reveals a number of early differentiating neurons as well as axon tracts, and ELAV expression is apparent within postmitotic neurons of all brain neuromeres, including the tritocerebrum. (C,D) By contrast, in *vnd*-null mutants, a gap is seen in the area of the tritocerebral region and the majority of ELAV-expressing cells are lacking in this domain. (E,F) Ubiquitous block of apoptosis partially restores the gap-like defects observed in *vnd* loss-of-function mutants: FAS2-immunoreactive longitudinal connectives are detectable, although neural fibres display fasciculation defects, and a significant number of ELAV-expressing cells are detectable in the cell death prevented *vnd* mutant tritocerebrum (F, arrow; compare with B).

maintenance. Thus, early in neurogenesis, a ventral subset of tritocerebral neuroblasts are lacking in *vnd* mutants, suggesting that *vnd* is required for the formation of neuroblasts in the developing tritocerebrum. Later in embryogenesis, *vnd* mutants display a severe loss of neural tissue, together with axonal patterning defects in the tritocerebrum. This gap-like phenotype is associated with increased apoptotic activity, and blocking apoptosis in *vnd*-null mutant embryos results in partial restoration of tritocerebral cells and axon tracts.

Together, these data suggest that, similar to its role in development of the embryonic VNC (Jimenez et al., 1995; Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002), *vnd* activity is required for proper formation and identity specification of tritocerebral neural lineages. Accordingly, our observation of increased apoptotic activity in the *vnd* mutant tritocerebrum might be a consequence of identity changes imposed on *vnd* deficient neural lineages. Indeed, *vnd* loss-of-function embryos are characterized by the ectopic expression of dorsal marker genes such as Ems and Msh in residual ventral neuroblasts of the *vnd* mutant tritocerebrum (Urbach et al., 2006). It is therefore conceivable that ectopic activity of genes such as Ems and Msh, together with the absence of *vnd* activity ultimately impose identity changes on residual tritocerebral *vnd* mutant lineages that are incompatible with their survival and eventually lead to cell death. In this sense, the gap-like brain phenotype observed in late *vnd* mutant embryos can be regarded as a consequence both of defects in neuroblast formation and of improper identity specification of tritocerebral neural lineages during embryonic brain development.

Integrated action of *vnd* and *lab* in embryonic brain development

Our results indicate that the homeotic gene *lab*, which is part of the anteroposterior patterning system, and the columnar gene *vnd*, which is involved in dorsoventral patterning, act in an integrated manner but independently in the formation and specification of the

tritocerebral neuromere. Although *vnd* and *lab* show overlapping expression in tritocerebral neuroblasts (Urbach and Technau, 2003b; Urbach and Technau, 2004) and subsequently in neural cells of the posterior tritocerebrum (this study), expression of *vnd* appears unaffected in *lab* mutant cells. Conversely, *vnd* does not act on *lab* expression as the complete absence of *lab* expression in *vnd* mutants (with the exception of a rare thin strand of neuronal cells) reflects a secondary defect because of the absence of cells that normally express *lab*. This independent genetic activity of *vnd* and *lab* is further supported by the fact that blocking apoptosis restores tritocerebral *lab* expression in *vnd*-null mutant embryos.

Thus, although the *lab* and *vnd* mutant brain phenotypes result in comparable axonal patterning defects (loss of the tritocerebral commissure and perturbation of the longitudinal connectives that normally run through this neuromere), their mode of action within the developing tritocerebrum is discriminable. Our results suggest that *vnd* is required for the specification of neural lineages within the developing tritocerebral neuromere, whereas the Hox gene *lab* appears to be independently required for the specification of neuronal identity within the same territory during later stages (Hirth et al., 1998; Page, 2000; Hirth et al., 2001; Sprecher et al., 2004). This indicates that the activity of the columnar gene *vnd* is integrated into pattern formation along the anteroposterior neuraxis by ensuring proper formation and development of tritocerebral neural lineages that subsequently become further specified by the activity of the Hox gene *lab*.

vnd/Nkx2 genes in brain development and evolution

The *Drosophila* columnar gene *vnd* belongs to the highly conserved *Nkx2* class of transcription factors that have been found in various animals, including mammals (Harvey, 1996; Cornell and von Ohlen, 2000). Notably, the *vnd/Nkx2* family of genes is exceptionally well conserved, both in terms of expression and function. Thus, the vertebrate homologues of *vnd* are expressed in the neural plate, or tube, in topologically similar positions as is *vnd* in the *Drosophila* ventral neuroectoderm and in the absence of *vnd/Nkx2* genes, ventral-most cells in the spinal cord and the *Drosophila* VNC are missing or transformed (Cornell and von Ohlen, 2000; Rallu et al., 2002). Moreover, this evolutionary conservation in expression and function of *vnd/Nkx2* genes appears to apply to some extent to brain development. A comparison of the anteroposterior order of *vnd/Nkx2* gene expression in the early embryonic brains of *Drosophila* and mouse reveals remarkable similarities (Urbach and Technau, 2003b; Urbach and Technau, 2004).

In terms of function, genetic knockouts in mice have shown that *Nkx2* genes appear to play a crucial role in patterning and neuronal specification during embryonic development of the telencephalon and hindbrain. *Nkx2.1* mutant mice display numerous brain patterning defects: the entire pituitary is missing (Kimura et al., 1996); the number of cortical interneurons is halved; there is a complete absence of TrkA-expressing cells in the developing telencephalon; and the ventral-most aspect of the telencephalon (the medial ganglionic eminence) becomes trans-fated to that of the adjacent more dorsolateral ganglionic eminence (Sussel et al., 1999). Thus, comparable with the role of *vnd* during *Drosophila* brain development (this study) (Urbach et al., 2006), *Nkx2.1* is involved in pattern formation and in cell fate determination during embryonic brain development in mice (Rallu et al., 2002).

In addition, recent studies have shown that *Nkx2.2* is involved in neural lineage specification in the developing hindbrain. In particular, the sequential generation of visceral motoneurons and serotonergic neurons from a common pool of neural progenitors located in the ventral hindbrain crucially depend on the integrated activities of Nkx2.2- and Hox1/2-class homeodomain proteins (Pattyn et al., 2003a; Pattyn et al., 2003b). An important function of these proteins is to coordinate the spatial and temporal activation of the homeodomain protein Phox2b, which in turn acts as a binary switch in the selection of motor neuron or serotonergic neuronal fate (Pattyn et al., 2003a; Samad et al., 2004). De-repressive activity of Nkx2.2 at or in vicinity of Pbx/Hox-binding sites proximal to the Phox2b enhancer enhances transcriptional activation of Phox2b by Hox1 and Pbx factors (Samad et al., 2004). These data suggest that comparable with the integrated activity of *vnd* and *lab* in *Drosophila* brain neuromere specification, integrated activity of the Nkx2.2 and Hox1/2 proteins is involved in the specification of segmental neural lineages. Thus, integration of anteroposterior and dorsoventral patterning systems by homeodomain transcription factors of the *Hox* and *vnd/Nkx2* genes might represent an ancestral feature of insect and mammalian brain development (Hirth et al., 2003).

We thank A. H. Brand, the Developmental Studies Hybridoma Bank, C. Q. Doe, P. Fichelson, R. Finkelstein, K. Matthews, D. M. Mellerick, J. B. Skeath, H. Vaessin and K. White for flies and antibodies. This work was supported by the Swiss NSF (to H.R.), the DFG (to R.U. and G.M.T.) and ELTEM-NEUREX (to F.M.R. and F.H.).

REFERENCES

Abrams, J. M., White, K., Fessler, L. I. and Steller, H. (1993). Programmed cell death during Drosophila embryogenesis. *Development* **117**, 29-43.
Bhat, K. M. (1999). Segment polarity genes in neuroblast formation and identity specification during *Drosophila* neurogenesis. *BioEssays* **21**, 472-485.
Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1992). deadpan, an essential pan-neural gene in Drosophila, encodes a helix-loop-helix protein similar to the hairy gene product. *Genes Dev.* **6**, 2137-2151.
Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
Campos-Ortega, J. A. and Hartenstein, V. (1997). *The Embryonic Development of Drosophila melanogaster*. Heidelberg: Springer.
Chu, H., Parras, C., White, K. and Jimenez, F. (1998). Formation and specification of ventral neuroblasts is controlled by vnd in Drosophila neurogenesis. *Genes Dev.* **12**, 3613-3624.
Cornell, R. A. and von Ohlen, T. V. (2000). Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol.* **10**, 63-71.
D'Alessio, M. and Frasch, M. (1996). msh may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Dev.* **58**, 217-231.
Harvey, R. P. (1996). *NK-2* Homeobox genes and heart development. *Dev. Biol.* **178**, 203-216.
Hirth, F., Therianos, S., Loop, T., Gehring, W. J., Reichert, H. and Furukubo-Tokunaga, K. (1995). Developmental defects in brain segmentation caused by mutations of the homeobox genes *orthodenticle* and *empty spiracles* in *Drosophila*. *Neuron* **15**, 769-778.
Hirth, F., Hartmann, B. and Reichert, H. (1998). Homeotic gene action in embryonic brain development of Drosophila. *Development* **125**, 1579-1589.
Hirth, F., Loop, T., Egger, B., Miller, D. F., Kaufman, T. C. and Reichert, H. (2001). Functional equivalence of Hox gene products in the specification of the tritocerebrum during embryonic brain development of Drosophila. *Development* **128**, 4781-4788.
Hirth, F., Kammermeier, L., Frei, E., Walldorf, U., Noll, M. and Reichert, H. (2003). An urbilaterian origin of the tripartite brain: developmental genetic insights from *Drosophila*. *Development* **130**, 2365-2373.
Isshiki, T., Takeichi, M. and Nose, A. (1997). The role of the *msh* homeobox gene during *Drosophila* neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development* **124**, 3099-3109.
Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.
Jimenez, F. and Campos-Ortega, J. A. (1990). Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of Drosophila melanogaster. *Neuron* **5**, 81-89.
Jimenez, F., Martin-Morris, L. E., Velasco, L., Chu, H., Sierra, J., Rosen, D. R.

and White, K. (1995). *vnd*, a gene required for early neurogenesis of Drosophila, encodes a homeodomain protein. *EMBO J.* **14**, 3487-3495.
Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10**, 60-69.
Klaes, A., Menne, T., Stollewerk, A., Scholz, H. and Klambt, C. (1994). The Ets transcription factors encoded by the Drosophila gene pointed direct glial cell differentiation in the embryonic CNS. *Cell* **78**, 149-160.
Lin, D. M. and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13**, 507-523.
McDonald, J. A., Holbrook, S., Isshiki, T., Weiss, J. B., Doe, C. Q. and Mellerick, D. M. (1998). Dorsoventral patterning in the *Drosophila* central nervous system: the *vnd* homeobox gene specifies ventral column identity. *Genes Dev.* **12**, 3603-3612.
Mellerick, D. M. and Nirenberg, M. (1995). Dorsal-ventral patterning genes restrict NK-2 homeobox gene expression to the ventral half of the central nervous system of *Drosophila* embryos. *Dev. Biol.* **171**, 306-316.
Mellerick, D. M. and Modica, V. (2002). Regulated vnd expression is required for both neural and glial specification in Drosophila. *J. Neurobiol.* **50**, 118-136.
Mergliano, J. and Minden, J. S. (2003). Caspase-independent cell engulfment mirrors cell death pattern in Drosophila embryos. *Development* **130**, 5779-5789.
Nassif, C., Daniel, A., Lengyel, J. A. and Hartenstein, V. (1998). The role of morphogenetic cell death during Drosophila embryonic head development. *Dev. Biol.* **197**, 170-186.
Page, D. T. (2000). labial acts to initiate neuronal fate specification, but not axon pathfinding, in the embryonic brain of Drosophila. *Dev. Genes Evol.* **210**, 559-563.
Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole mount *Drosophila* embryos and larvae using antibody probes. In *Methods in Cell Biology. Drosophila melanogaster: Practical Uses in Cell Biology*. Vol. 44 (ed. L. S. B. Goldstein and E. Fyrberg), pp. 445-487. New York: Academic Press.
Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.
Pattyn, A., Vallstedt, A., Dias, J. M., Samad, O. A., Krumlauf, R., Rijli, F. M., Brunet, J. F. and Ericson, J. (2003a). Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. *Genes Dev.* **17**, 729-737.
Pattyn, A., Vallstedt, A., Dias, J. M., Sander, M. and Ericson, J. (2003b). Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. *Development* **130**, 4149-4159.
Pereanu, W. and Hartenstein, V. (2004). Digital three-dimensional models of Drosophila development. *Curr. Opin. Genet. Dev.* **14**, 382-391.
Rallu, M., Corbin, J. G. and Fishell, G. (2002). Parsing the prosencephalon. *Nat. Rev. Neurosci.* **3**, 943-951.
Richter, S., Hartmann, B. and Reichert, H. (1998). The wingless gene is required for embryonic brain development in Drosophila. *Dev. Genes Evol.* **208**, 37-45.
Samad, O. A., Geisen, M. J., Caronia, G., Varlet, I., Zappavigna, V., Ericson, J., Goridis, C. and Rijli, F. M. (2004). Integration of anteroposterior and dorsoventral regulation of Phox2b transcription in cranial motoneuron progenitors by homeodomain proteins. *Development* **131**, 4071-4083.
Schmidt-Ott, U. and Technau, G. M. (1992). Expression of en and wg in the embryonic head and brain of Drosophila indicates a refolded band of seven segment remnants. *Development* **116**, 111-125.
Skeath, J. B. (1999). At the nexus between pattern formation and cell-type specification: the generation of individual neuroblast fates in the *Drosophila* embryonic central nervous system. *BioEssays* **21**, 922-931.
Skeath, J. B. and Thor, S. (2003). Genetic control of Drosophila nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.
Spana, E. P. and Doe, C. Q. (1995). The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila. *Development* **121**, 3187-3195.
Sprecher, S. G., Muller, M., Kammermeier, L., Miller, D. F., Kaufman, T. C., Reichert, H. and Hirth, F. (2004). Hox gene cross-regulatory interactions in the embryonic brain of Drosophila. *Mech. Dev.* **121**, 527-536.
Sussel, L., Marin, O., Kimura, S. and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**, 3359-3370.
Therianos, S., Leuzinger, S., Hirth, F., Goodman, C. S. and Reichert, H. (1995). Embryonic development of the Drosophila brain: formation of commissural and descending pathways. *Development* **121**, 3849-3860.
Tremml, G. and Bienz, M. (1992). Induction of labial expression in the Drosophila endoderm: response elements for dpp signalling and for autoregulation. *Development* **116**, 447-456.
Urbach, R. and Technau, G. M. (2003a). Molecular markers for identified neuroblasts in the developing brain of Drosophila. *Development* **130**, 3621-3637.
Urbach, R. and Technau, G. M. (2003b). Segment polarity and DV patterning gene expression reveals segmental organization of the Drosophila brain. *Development* **130**, 3607-3620.
Urbach, R. and Technau, G. M. (2004). Neuroblast formation and patterning during early brain development in Drosophila. *BioEssays* **26**, 739-751.
Urbach, R., Schnabel, R. and Technau, G. M. (2003). The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in Drosophila. *Development* **130**, 3589-3606.
Urbach, R., Volland, D., Seibert, J. and Technau, G. M. (2006). Segment-specific requirements for dorsoventral patterning genes during early brain development in Drosophila. *Development* **133**, 4315-4330.
von Ohlen, T. and Doe, C. Q. (2000). Convergence of *dorsal*, *dpp*, and *egfr* signaling pathways subdivides the *Drosophila* neuroectoderm into three dorsal-ventral columns. *Dev. Biol.* **224**, 362-372.
Weiss, J. B., Von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q. and Scott, M. P. (1998). Dorsoventral patterning in the *Drosophila* central nervous system: the *intermediate neuroblasts defective* homeobox gene specifies intermediate column identity. *Genes Dev.* **12**, 3591-3602.