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Segment-specific requirements for dorsoventral patterning genes during early brain development in *Drosophila*

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An initial step in the development of the *Drosophila* central nervous system is the delamination of a stereotype population of neural stem cells (neuroblasts, NBs) from the neuroectoderm. Expression of the columnar genes *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*) and *muscle segment homeobox* (*msh*) subdivides the truncal neuroectoderm (primordium of the ventral nerve cord) into a ventral, intermediate and dorsal longitudinal domain, and has been shown to play a key role in the formation and/or specification of corresponding NBs. In the procephalic neuroectoderm (pNE, primordium of the brain), expression of columnar genes is highly complex and dynamic, and their functions during brain development are still unknown. We have investigated the role of these genes (with special emphasis on the *Nkx2*-type homeobox gene *vnd*) in early embryonic development of the brain. We show at the level of individually identified cells that *vnd* controls the formation of ventral brain NBs and is required, and to some extent sufficient, for the specification of ventral and intermediate pNE and deriving NBs. However, we uncovered significant differences in the expression of and regulatory interactions between *vnd*, *ind* and *msh* among brain segments, and in comparison to the ventral nerve cord. Whereas in the trunk Vnd negatively regulates *ind*, Vnd does not repress *ind* (but does repress *msh*) in the ventral pNE and NBs. Instead, in the deutocerebral region, Vnd is required for the expression of *ind*. We also show that, in the anterior brain (protocerebrum), normal production of early glial cells is independent from *msh* and *vnd*, in contrast to the posterior brain (deuto- and tritocerebrum) and to the ventral nerve cord.

KEY WORDS: CNS, Brain, Neuroectoderm, Neuroblasts, Dorsoventral patterning, Cell fate, *vnd*, *msh*, *ind*, Glia, *Drosophila*

INTRODUCTION

The *Drosophila* central nervous system, which can be subdivided into ventral nerve cord (VNC) and brain, provides a useful model with which to study the generation of neural cell diversity and patterning. It develops from bilaterally symmetrical neuroectoderm, which gives rise to a fixed number of neural stem cells, called neuroblasts (NBs). NBs, which form the VNC, descend from the truncal region of the neuroectoderm, whereas those that form the brain derive from the procephalic neuroectoderm (pNE) (Hartenstein and Campos-Ortega, 1984; Poulson, 1950). The developmental processes that underlie NB formation, cell fate specification and pattern formation have been extensively studied in the embryonic VNC. In early stages of embryogenesis, each segment of the truncal neuroectoderm is subdivided by segment-polarity genes into transverse rows along the anteroposterior axis, and by 'columnar genes' (DV patterning genes) into longitudinal columns along the dorsoventral axis. The superimposition of their gene activities establishes a Cartesian coordinate system of positional cues conferring specific identities to neural equivalence groups, of which each gives rise to a single NB (reviewed by Bhat, 1999; Skeath, 1999; Skeath and Thor, 2003). Accordingly, each NB acquires a unique identity, which is reflected by the time and position of its delamination from the neuroectoderm, by the combination of genes it expresses, and by the production of a specific cell lineage (e.g. Bossing et al., 1996; Broadus et al., 1995; Doe, 1992; Schmidt et al., 1997). Through the expression of proneural genes, each cell of an equivalence group (or proneural cluster) acquires the potential to become a NB (e.g. Skeath and Carroll, 1992). Notch signaling ensures that only a single cell adopts a NB fate and the others assume an epidermal fate (reviewed by Campos-Ortega, 1995).

The truncal neuroectoderm is subdivided along the dorsoventral axis into adjacent longitudinal columns by the expression of three genes: *ventral nervous system defective* (*vnd*) in the ventral, *intermediate neuroblasts defective* (*ind*) in the intermediate and *muscle segment homeobox* [*msh*; *Drop* (*Dr*) – FlyBase] in the dorsal neuroectodermal column (Buescher and Chia, 1997; Chu et al., 1998; D'Alessio and Frasch, 1996; Isshiki et al., 1997; Jimenez et al., 1995; Mellerick and Nirenberg, 1995; Weiss et al., 1998). Experimental data provide evidence that these homeobox genes interact in a hierarchical cascade of transcriptional repression, which is necessary to establish and maintain the sharp boundaries between their domains of expression. According to that hierarchy, *vnd* represses *ind* (and *msh*) in the ventral column, and *ind* represses *msh* in the intermediate column (reviewed by Cornell and Von Ohlen, 2000; Skeath, 1999).

vnd, the prototype of the *Nkx2*-type gene family, is expressed not only in the ventral column neuroectoderm, but also in descending NBs, and is maintained in subsets of their daughter cells. Coincident with its pattern of expression, *vnd* is required for specification of ventral column neuroectoderm, as well as for formation and specification of ventral NBs and their progeny (Chu et al., 1998; Jimenez and Campos-Ortega, 1990; McDonald et al., 1998; Mellerick and Nierenberg, 1995; Mellerick and Modica, 2002; Shao et al., 2002; Skeath et al., 1994). Similar roles are fulfilled by *ind* in intermediate, and *msh* in dorsal, column neuroectoderm and NBs, although *msh* is dispensable for the formation of dorsal NBs (Isshiki et al., 1997; Weiss et al., 1998). Owing to much higher complexity and hidden segmental organization, the mechanisms that control pattern formation in the brain are largely unknown. The brain can be subdivided (from posterior to anterior) into the tritocerebrum, deutocerebrum and protocerebrum, which derive from the intercalary,

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antennal and ocular (plus labral) segments, respectively (Schmitt-Ott and Technau, 1992) (reviewed by Urbach and Technau, 2003a). Recently, we have described the pattern of the entire population of brain NBs (Urbach et al., 2003) and could show that, based on the combination of genes expressed, each brain NB acquires a unique identity (Urbach and Technau, 2003b). Furthermore, we have presented first data on the expression of the columnar genes in the early embryonic brain (Urbach and Technau, 2003c).

In this study, we have investigated the function of columnar genes during early brain development with the main focus on *vnd*. We show that *vnd* is required for formation of ventral brain NBs, and that cell death contributes to the loss of brain NBs in embryos lacking *vnd* function. Detailed analysis of the expression of an array of molecular markers in loss- and gain-of-function backgrounds indicates that *vnd* is also necessary, and to some extent sufficient, for the proper specification of ventral and intermediate pNE and brain NBs. We further demonstrate that control of *ind* and *msh* expression by *vnd* differs among brain neuromeres and between brain and VNC. Finally, we show that the generation of normal numbers of early glial cells in the protocerebrum is independent of *msh* and *vnd*, which is in contrast to the situation in the trito-/deutocerebrum and the VNC.

MATERIALS AND METHODS

Drosophila strains

The following fly strains were used: Oregon R (wild type), *engrailed-lacZ* (ryXho25) (Hama et al., 1990), *svp-lacZ (H162)* (Mlodzik et al., 1990), vnd^{6} (Jimenez and Campos-Ortega, 1990) and msh^{d68} (Isshiki et al., 1997). The UAS-Gal4 system (Brand and Perrimon, 1993) was used to overexpress *vnd* in the neuroectoderm, crossing UAS-*vnd* (Chu et al., 1998) with the *sca*-Gal4 driver line (Klaes et al., 1994).

Staging, flat preparation, and mounting of embryos

Staging of the embryos was carried out according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997). Flat preparations of the head ectoderm of stained embryos and mounting were carried out as described previously (Urbach et al., 2003).

Antibodies and immunohistochemistry

Embryos were dechorionated, fixed and immunostained according to previously published protocols (Patel, 1994). The following primary antibodies were used: rabbit-anti-Atonal [1:5000 (Jarman et al., 1993); kindly provided by A. Jarman], rabbit anti-cleaved human caspase 3 [1:50 (Brennecke et al., 2003); Cell Signaling Technology], mouse-anti-Dachshund [1:250 (Mardon et al., 1994); DSHB], rabbit-anti-Deadpan [1:300 (Bier et al., 1992); kindly provided by H. Vaessin], anti-DIG-AP (1:1000, Roche), rat-anti-Empty spiracles [1:1000 (Walldorf and Gehring, 1992); kindly provided by U. Walldorf], mouse-anti-Engrailed [4D9,1:7 (Patel et al., 1989); DSHB], rabbit-anti-Eyeless (1:1000, kindly provided by U. Walldorf), mouse-anti-β-Galactosidase (1:500, Promega), rabbit-anti-β-Galactosidase (1:2500, Cappel), rat-anti-Gooseberry-distal [16F12 and 10E10, 1:2 (Zhang et al., 1994); kindly provided by B. Holmgren], mouse-anti-Ladybird early [1:2 (Jagla et al., 1997); kindly provided by K. Jagla], rabbit-anti-Muscle segment homeobox (1:500; kindly provided by M. P. Scott), rabbit-anti-Repo (1:500) (Halter et al., 1995), rat-anti-Sloppy paired [1:300 (Cadigan et al., 1994); kindly provided by W. Gehring], rabbit-anti-Ventral nervous system defective [1:2000 (McDonald et al., 1998); kindly provided by C. Doe] and mouse-anti-Wingless (1:10, DSHB). The secondary antibodies (donkey anti-mouse, goat anti-rabbit; donkey anti-rat; Dianova) were either biotinylated or alkaline phosphatase-conjugated and diluted 1:500.

Whole mount in situ hybridization

DIG-labeled *intermediate neuroblast defective* RNA probe (kindly provided by M. P. Scott) was synthesized using *Hin*dIII linearized pNB40-ind as a template with T7 polymerase, and DIG-labeled *lethal of scute* RNA probe was synthesized using the EST clone lsc RE59335 (BDGP) cloned in *Pst*I linearized pFLC1 as a template with T3 polymerase according to the manufacturers protocol (Roche). The hybridization on embryos was performed as described previously (Plickert et al., 1997; Tautz and Pfeifle, 1989).

Documentation

Embryos were viewed under a Zeiss Axioplan equipped with Nomarski optics using 40×, 63× and 100× oil immersion objectives. Pictures were digitized with a CCD camera (Contron progress 3012) and different focal planes were combined using Adobe Photoshop 7.0. Semi-schematic presentations are based on camera lucida drawings.

RESULTS

Expression of Vnd during early embryonic brain development is highly dynamic

Expression of Vnd protein is first detectable in the blastoderm in bilateral longitudinal columns along the ventral neuroectoderm of the trunk and the ventral procephalic neuroectoderm (pNE), covering the prospective ventral parts of the trito- (TC), deuto- (DC) and protocerebrum (PC; Fig. 1A). Although in the trunk Vnd expression is maintained within a continuous ventral neuroectodermal column during subsequent stages, it becomes more complex and diverse among head segments. By stage 8 [before first brain neuroblasts (NBs) have developed], Vnd becomes downregulated in parts of the procephalic domain (Fig. 1B). Until stage 10, Vnd has largely vanished in the anterior pNE and NBs of the DC, but its level remains high in the ventral pNE and delaminating NBs of the TC, posterior DC and PC (Fig. 1C-E). During stages 10/11, Vnd becomes downregulated at the ventral border between TC and DC (Fig. 1F-K). Accordingly, by late stage 11, Vnd expression is restricted to separate domains at the posterior border of the TC, DC and PC, respectively (Fig. 1I-K). Whereas the number of Vnd-positive NBs in the domains of the TC and DC is rather small, a large population of about 13 NBs is found in the PC (Fig. 1J,K) (see also Urbach and Technau, 2003c).

Thus, in contrast to the situation in the trunk, Vnd expression in the early brain is highly dynamic and becomes progressively confined to three separate ventral domains, encompassing different numbers of NBs and their progeny in the posterior compartments of the TC, DC and PC [for Vnd expression at later stages, see Sprecher et al. (Sprecher et al., 2006)].

Absence of Vnd affects formation of ventral brain NBs

To assay the presence of individual brain NBs in embryos lacking *vnd* function (referred to as '*vnd* embryos'), we used morphological criteria (size and position), the NB marker Deadpan (Dpn) and Seven up-*lacZ* (Svp-*lacZ*). Between stages 9 and 11 Svp-*lacZ*-positive brain NBs represent stable reference points, also allowing identification of Svp-*lacZ*-negative NBs surrounding them (Urbach et al., 2003). We focused mainly on the pattern of NBs at stage 9 (corresponding to the S2 NB pattern in the ventral nerve cord, VNC), and at stage 11 (corresponding to the S5 NB pattern in the VNC) (Doe, 1992) when the full complement of brain NBs is established (Urbach et al., 2003). The expression of all molecular markers used in this study for the identification of individual NBs in wild-type and mutant embryos is summarized in Fig. 2 and Table 1. The analysis of large samples of embryos allowed us to quantify the data and thus to determine the variability of the phenotypes. The frequency at which each NB is found in *vnd* embryos is indicated in percent in Fig. 2B′,D′.

In contrast to the PC and DC and the neuromeres of the VNC, NB formation in the TC initiates after stage 9. In the PC and DC of stage 9 *vnd* embryos almost all ventral brain NBs are improperly specified

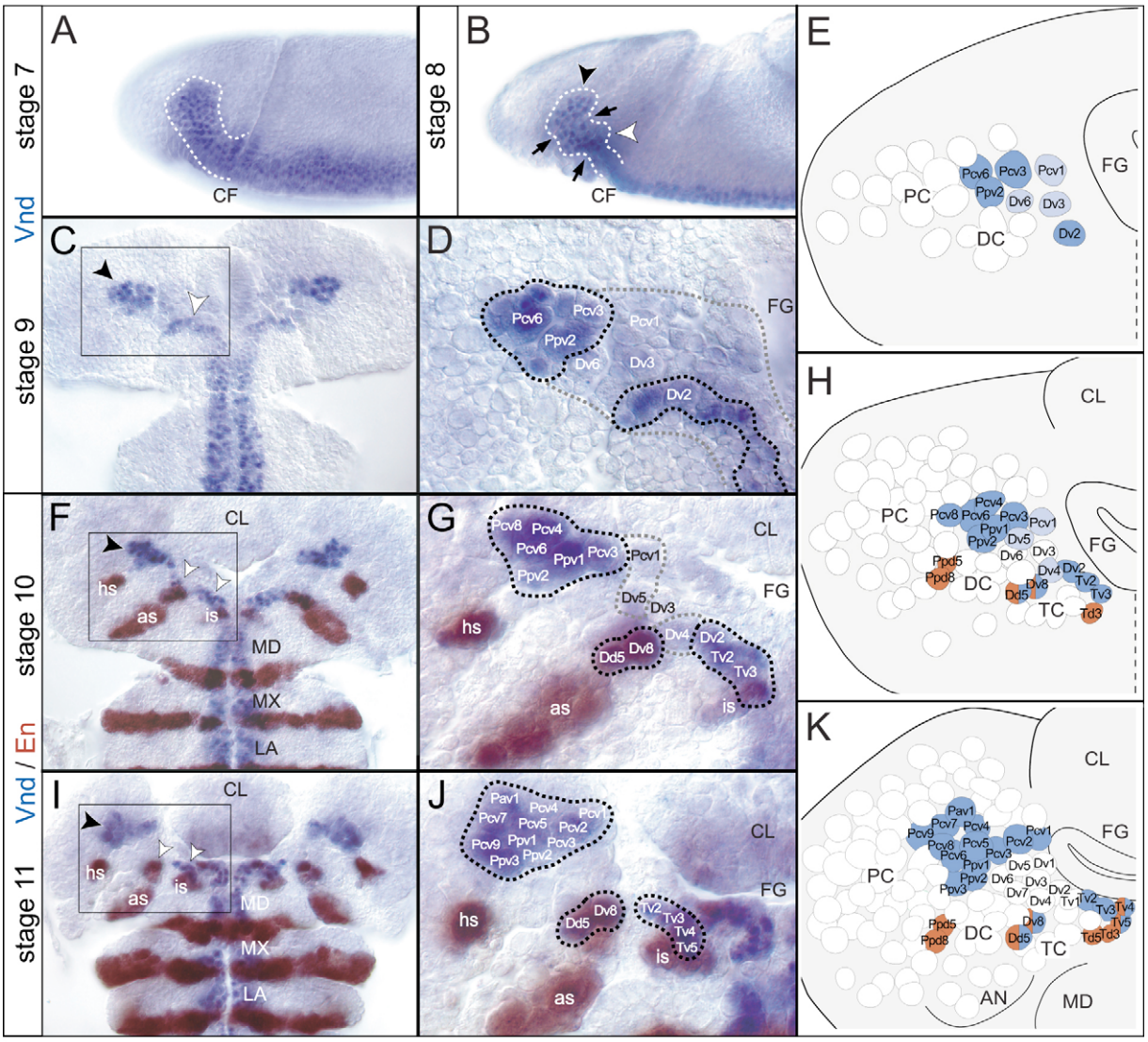

Fig. 1. Expression of Vnd in the pNE and brain NBs of early wild-type embryos. (A-K) Vnd antibody staining at early stage 7 (A), stage 8 (B), stage 9 (C,D,E), stage 10 (F,G,H) and stage 11 (I,J,K). (F-K) Double labeling against Engrailed-*lacZ* (En) indicates part of the posterior border of brain neuromeres. (C,F,I) Flat preparations displaying the head ectoderm (anterior is upwards). (D,G,J) Higher magnifications of regions framed in C,F,I focusing on NBs; immuno-positive NBs are indicated by white letters, immuno-negative NBs are indicated by black letters. Broken black lines encircle domains of strong levels of Vnd; grey lines circle weak levels of Vnd. (E,H,K) Expression of Vnd and En in brain NBs (dark blue, strong Vnd; light blue, weak Vnd; brown, En). All NBs developing within the Vnd domain have been assigned as ventral NBs [for nomenclature of NBs, see Urbach et al. (Urbach et al., 2003)]. (**A**,**B**) Lateral view of anterior half of whole-mount embryos. Broken white line encircles the procephalic Vnd domain, encompassing about 66 cells (A, stage 7) and about 47 cells (B, stage 8), indicating that Vnd is already downregulated in part of the pNE cells (black arrows). The prospective protocerebral Vnd domain (black arrowhead) begins to separate from the trito-/deutocerebral Vnd domain (white arrowhead). (**C-E**) Vnd has mostly vanished from the anterioventral pNE of the deutocerebrum (DC) and posterioventral pNE of the protocerebrum (PC), and from the ventral NBs Dv3, Dv6 and Pcv1. (**F-H**) Vnd begins to vanish in the posterior DC and anterior tritocerebrum (TC; area around Dv4 in G). (**I-K**) Separate Vnd domains are located at the posterioventral border of the TC, DC and PC. as, *en* antennal stripe; hs, *en* head spot; is, *en* intercalary stripe; AN, MD, MX, LA, antennal, mandibular, maxillary, labial segment, respectively; CL, clypeolabrum; CF, cephalic furrow; FG, foregut.

(see below) or missing. Penetrance of the effects significantly differs among the individual NBs (Fig. 2B′). For example, Dv2 (27%) fails to form about three times more often than the neighboring Dv3 [90%; for nomenclature of brain NBs, see Urbach et al. (Urbach et al., 2003)].

By stage 11, a lack of NBs becomes more apparent in the ventral domains of the TC, DC and most prominently the PC of *vnd* embryos stained with Dpn antibody (Fig. 3A-D). Svp-*lacZ* in wild type is expressed in about two thirds of the 26 ventral brain NBs (Fig. 3E). In *vnd* mutants, all ventral Svp-*lacZ*-positive, as well as the surrounding immunonegative, NBs are affected, but to different degrees (Fig. 2D,D′, Fig. 3F). By contrast, all dorsal brain NBs can be identified in *vnd* mutants, including Dd5, the only dorsal brain NBs that, in wild type, transiently co-express *msh* and *vnd*.

Taken together, a considerable proportion of ventral NBs is not formed in the TC, DC and PC of *vnd* embryos. The probability of being part of this fraction significantly differs among the individual NBs.

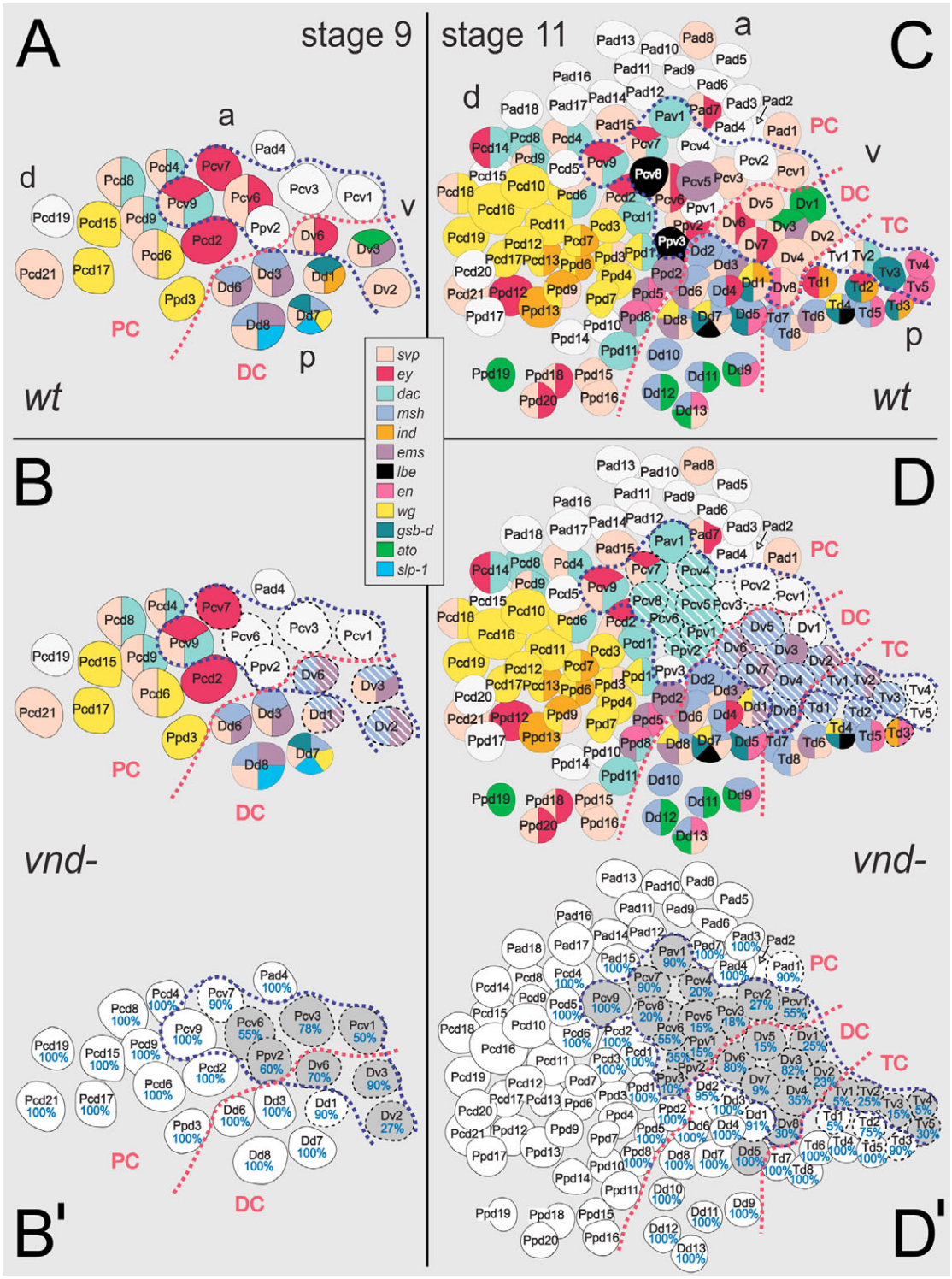

Fig. 2. Summary diagram of marker genes expressed in brain NBs of wild-type and *vnd* mutant embryos at stages 9 and 11. (**A-D**) The schemes represent NB maps of one hemibrain: anterior (a) is towards the top, posterior (p) is towards the bottom, dorsal (d) is leftwards, ventral (v) is rightwards. Numbers and distribution of NBs correspond to the situation in flat preparations of wild-type embryos at stage 9 (A,B,B′) and stage 11 (C,D,D′). The color code indicates expression of the genes investigated in this study in individual brain NBs. Broken blue lines enclose the domain of Vnd expression and, accordingly, the population of ventral NBs; broken red lines mark neuromeric boundaries between TC, DC and PC. (A,C) Wild type (*wt*). (B,B′,D,D′) *vnd* embryos (*vnd*-); individual NBs encircled with broken black lines are absent to a certain degree. (B′,D′) The frequencies of their presence (in %) are indicated at stage 9 for all NBs, at stage 11 for all NBs in the TC, DC and all ventral and adjacent dorsal NBs in the PC. NBs (grey) develop within the Vnd domain. (B,D) Expression of a marker is indicated as missing if it is not detected in more than 90% observed cases. Hatched colors indicate ectopic gene expression. As the exact identity of NBs that ectopically express *msh*, *ems* and *dac* is not clear, hatched NBs represent potential candidates, of which only a subfraction will normally be found to express the respective marker. For further details, see text.

Cell death contributes to loss of ventral brain NBs in *vnd* mutant embryos

In order to investigate whether cell death accounts for loss of ventral brain NBs, we performed antibody staining against activated caspase 3 (Casp3). Casp3 activation is one of the early hallmarks of cell death. In the wild-type pNE, cell death first becomes detectable by late stage 10, and increases rapidly during stages 11/12 (see also Nassif et al., 1998). A comparable time course is observed in *vnd* embryos. However, apoptosis is significantly more pronounced in the ventral pNE of the developing DC and PC, but not TC (Fig. 4A,B). During early/middle stage 11, Casp3 staining was found in cells of the pNE and in brain NBs (Fig. 4C), at relative positions corresponding to the loss of deuto- and protocerebral NBs at later stages (compare Fig. 4C with 4D,E). We did not detect apoptotic NBs in the TC. These data suggest that apoptosis of neuroectodermal progenitor cells, and of already developed NBs in the DC and PC, partially accounts for the defects in the pattern of brain NBs in *vnd* embryos [for apoptosis in *vnd* mutant brain at later stages, see Sprecher et al. (Sprecher et al., 2006)].

Absence of Vnd affects the specification of ventral pNE and residual ventral and intermediate brain NBs

As shown above, the ventral pNE of *vnd* embryos produces a reduced set of brain NBs. In order to uncover the identity of residual NBs at stage 11, we analyzed the expression of marker genes, which are normally exclusively (Muscle segment homeobox, Msh) or preferentially (Empty spiracles, Ems; Dachshund, Dac; Ladybird early, Lbe; Eyeless, Ey) expressed in dorsal pNE and specific subsets of dorsal NBs (for wild-type marker gene expression, see Fig. 2A,C) (see also Urbach and Technau, 2003b). The combinations and frequencies in which the various markers are expressed in individual brain NBs of *vnd* embryos are summarized in Fig. 2B,D and in Table 1. In wild type, Msh is expressed in the dorsal pNE of the TC and DC, but not of the PC (Fig. 6A-D). In *vnd* embryos, Msh is derepressed in the ventral pNE and residual ventral NBs in the TC and DC, but again not detected in the PC (Fig. 6E-H). In *vnd* mutants, Ems expression becomes ectopically expanded into large parts of the ventral antennal and intercalary pNE, and into part of the residual ventral NBs (three to four in the DC and one in the TC; Fig. 5A-D), resembling the pattern of ectopic Msh. Lbe expression in the pNE of the TC (and slightly in the DC) is expanded ventrally, although we did not detect descending ectopic Lbe-positive NBs (Fig. 5E-H). Dac is derepressed in part of the ocular Vnd mutant domain (Fig. 5I-L), and we identified Dac in two to four additional ventral NBs in the PC descending from this ectopic Dac domain (Fig. 5L). Furthermore, Ey is lacking in intermediate pNE (and respective NBs) of the TC, but is ectopically expressed in the ventral pNE (Fig. 5M-P). These data indicate that the expression of several dorsal marker genes is expanded in *vnd* mutants into the ventral pNE and deriving NBs, suggesting that loss of Vnd at least in part leads to a ventral-to-dorsal transformation of ventral pNE and residual ventral NBs.

To find out whether 'intermediate NBs', which normally derive from the insulated domains of *ind* expression (e.g. Td1, Td2, Td3 and Dd1), are also affected in the mutant, we investigated the expression of *intermediate neuroblasts defective* (*ind*), Ey, Gooseberry-distal (Gsb-d), Wingless (Wg) and Svp-*lacZ*, markers which are co-expressed in part of these precursors in wild type (see Fig. 2A,C, Table 1) (Urbach and Technau, 2003b). In *vnd* embryos, Td3 in most cases develops properly (Fig. 2D, Fig. 3D, Fig. 6T). By contrast, lack of *ind* is found in positions of Td1, Td2 and Dd1 (Fig. 2D, compare Fig. 6P with 6T). Expression of the other marker genes suggests that Td2 and Dd1 are mostly formed but become improperly specified, whereas Td1 is not formed (Fig. 2D, Fig. 3D).

Together, these data indicate that Vnd is required for proper specification of ventral pNE and residual ventral, as well as for intermediate NBs.

Ectopic Vnd changes the identity of dorsal pNE and dorsal brain NBs

To test whether *vnd* is able to alter the fate of dorsal pNE and NBs, we ectopically expressed Vnd (using *sca*-Gal4; embryos in the following termed *sca-vnd*) and examined the expression of Dac, Ems, En, Ey, Lbe, Msh and Wg, which are specifically expressed in dorsal and/or ventral parts of the pNE and brain NBs. In *sca-vnd* embryos, these markers are largely suppressed or completely absent in the dorsal pNE and NBs of the TC (Ems, Ey, Lbe, Msh and Wg), DC (Ems, En, Ey, Lbe, Msh, and Wg) and PC (Dac, Ems, En, Ey, and Wg) (Fig. 5Q-X, Fig. 6I-L and data not shown). The effects of ectopic Vnd on the level of individual NBs are summarized in Table 1. Susceptibility to ectopic Vnd significantly differs among dorsal NBs in a cell-specific manner. For example, Lbe is frequently missing in the dorsal Dd7 (40%), and always in the dorsal Td4 (0%). The two ventral Lbe-positive NBs in the PC, Ppv3 and Pcv8, are maintained (100%), and, in addition, we usually identified ectopic Lbe in one to six adjacent NBs in the dorsal PC (compare Fig. 5F with 5T). This indicates that ectopic Vnd suppresses Lbe in the dorsal TC and DC, but confers ventral fates to a subset of dorsal NBs in the PC.

Taken together, in the *vnd* gain-of-function situation, we recognized a wide-ranging loss of specific marker gene expression and a partial dorsal-to-ventral transformation of dorsal pNE and corresponding brain NBs.

Regulation of *ind* and *msh* by Vnd in the pNE and brain NBs differs from the trunk in a segment-specific manner

In wild type, the expression pattern of *ind* and *msh* differs between head and trunk and among head segments. *ind* is expressed in three separate pNE patches, of which the intercalary and antennal *ind* spot are located at intermediate position between the dorsal Msh and ventral Vnd domain. The domains of Msh and Vnd share a common border at sites lacking an intervening *ind* domain. In the ocular segment, the *ind* domain is spatially separated from the Vnd domain, and Msh is not expressed (Fig. 6A-D,M-P) (see also Urbach and Technau, 2003c). To assay whether *vnd* regulates these DV patterning genes in the pNE and brain NBs, we examined the expression of Msh and *ind* in *vnd* loss-of-function and gain-of-function embryos.

In *vnd* mutants, *ind* is derepressed within the ventral part of the truncal neuroectoderm (Weiss et al., 1998). Surprisingly, we did not observe such a derepression of *ind* in the ventral intercalary and antennal segment (compare Fig. 6M,O with 6Q,S). Instead, expression of *ind* in the intercalary segment is delayed (starting after stage 9) and found in a smaller area. Even more unexpectedly, *ind* in the antennal pNE and Dd1 was never detected [0%, *n*=30; 'cluster1' in McDonald et al. (McDonald et al., 1998)]. This indicates that in the antennal segment, Vnd is necessary for activating *ind*, rather than for repressing it. *ind* transcript in the ocular pNE often appears slightly expanded ventrally (70%, *n*=30; Fig. 6O,S).

Table 1. Expression of marker genes specific for NB subsets in wt, *vnd* loss-of-function (*vnd–*) and gain-of-function (*sca-vnd*) embryos at stage 11

	Wild type*			*vnd–*						*sca-vnd*					
	d	i	v	d	%	i	%	v	%	d	%	i	%	v	%
Msh		–	–					***Dv2***	***23***			–		–	
								Dv3	***82***						
								Dv4	***35***						
								Dv5	***15***						
	Dd2-13			Dd2-13	100	***Dd1***	***91***	***Dv6***	***80***	**Dd2-13**	**0**				
								Dv7	***9***						
								Dv8	***30***						
	Td4-8			Td4-8	100	***Td2***	***75***	***Tv1,4***	***5***	**Td4-8**	**0**				
								Tv2	***25***						
								Tv3	***15***						
								Tv5	***30***						
ind	–	(Ppd6,9,13 Pcd7,13)†	–	–		(Ppd6,9,13 Pcd7,13)†	All 100	–		–		**2-3 PCNBs**		**–**	
		Dd1				**Dd1**	**0**					**Dd1**	**92**		
		Td1-3				**Td1,2**	**0**					**Td1-3**	**0**		
						Td3	**85**								
Ey	Pad7		Pcv6	Pad7				**Pcv6**	**55**	**Pad7**				Pcv6	100
	Pcd2,14		Pcv7	Pcd2,14				Pcv7	95	**Pcd2,14**				Pcv7	100
	Ppd12,18, 20		Pcv9	Ppd12,18, 20				Pcv9	100	**Pcd12,18, 20**				Pcv9	100
			Ppv2					**Ppv2**	**35**					**Ppv2**	**55**
	Dd4	Td1	Dv6	Dd4		**Td1**	**0**	**Dv6**	**0**	**Dd4**		**Td1**	**0**	Dv6	100
		Td2	Dv7		All 100	**Td2**	**40**	**Dv7**	**10**		**All <5**	**Td2**	**0**	**Dv7**	**20**
Dac	Pcd1,2,4, 6,8,9,14	–	Pav1	Pcd1,2,4, 6,8,9,14		–		Pav1	100	**Pcd1,2,4, 6,8,9,14**	**<20**	–		**Pav1**	**40**
			Pcv7					Pcv7,9	100					Pcv7	100
	Ppd1,9,11		Pcv9	Ppd1,9,11				***2-4 PCNBs***		**Ppd1,9,11**	**<20**			Pcv9	100
			Tv2		All 100			**Tv2**	**25**	Pcd2	100			Tv2	95
Ems	Ppd2,5,8	–	Pcv5	Ppd2,5,8				**Pcv5**	**15**	**Ppd2**	**30**	–		**Pcv5**	**88**
										Ppd5	**0**				
										Ppd8	**20**				
	Dd3,6,8		Dv3	Dd3,6,8		***Dd1***	***80***	**Dv3**	**85**	**Dd3**	**12**			Dv3	100
								Dv2	***20***	**Dd6**	**87**				
				Td6				***Dv6***	***70***	**Dd8**	**92**				
	Td6				All 100			***Tv2***	***25***	**Td6**	**0**				
Lbe		–	Pcv8			–		**Pcv8**	**0**	***1-6 PCNBs***		–		Pcv8	100
	Dd7		Ppv3	Dd7				**Ppv3**	**0**	**Dd7**	**40**			Ppv3	100
	Td4			Td4	All 100					**Td4**	**0**				
En	Ppd5,8		Dv8	Ppd5,8				**Dv8**	**30**	**Ppd5**	**75**				
	Dd5		Tv4,5	Dd5				**Tv4**	**5**	**Ppd8**	**30**				
	Td5	Td3		Td5		**Td3**	**90**	**Tv5**	**30**	**Dd5**	**25**			**Dv8**	**75**
					All 100					Td5	100	Td3	100	Tv4,5	100
Gsb-d	Ppd3,10			Ppd3,10						n.d.		n.d.		n.d.	
	Dd5,7			Dd5,7		**Td2**	**75**								
	Td4	Td2,3	Tv3	Td4	All 100	**Td3**	**75**	**Tv3**	**11**						

Table 1 continued on next page.

Table 1. Continued

	Wild type*			*vnd–*						*sca-vnd*					
	d	i	v	d	%	i	%	v	%	d	%	i	%	v	%
Wg	Pcd3,6,7, 10-13, 16-19 Ppd1,3,4, 6,7,9 Dd1,7,8 Td4	–	–	Pcd3,6,7, 10-13, 16-19 Ppd1,3,4, 6,7,9 Dd1,7,8 Td4	All 100	–		–		**Pcd3,6,7, 10-13, Pcd16-19 Ppd1,3,4, 6,7,9 Dd1,7,8 Td4**	**10 10 90 10 10 45 35**	–		–	
Svp-lacZ	Pad1,7,8, 15 Pcd2,4,6, 9,18,21 Ppd3,9,15, 16,18,20	Dd1	Pcv1,3, 6,7,9 Ppv2	Pad1,7,8, 15 Pcd2,4,6, 9,18,21 Ppd3,9,15, 16,18,20		**Dd1**	**90**	**Pcv1** Pcv3 Pcv6 Pcv7 Ppv2 Pcv9 Ppv2 Dv2	**85** **18** **55** **85** **25** 100 **18** **27**	n.d.		n.d.		n.d.	
	Dd3,6,7,8, 13 Td6,8		Dv2-8 Tv2	Dd3,6,7,8, 13 Td6,8	All 100			**Dv3,6 Dv4,8 Dv5 Dv7 Tv2**	**82 35 15 9 25**						
Ato	Ppd19	–		Ppd19		–		**Dv1**	**25**	n.d.		n.d.		n.d.	
	Dd9,11, 12,13		Dv1,3	Dd9,11, 12,13	All 100			**Dv3**	**5**						

Percentages indicate presence of marker in *vnd–* or *sca-vnd* (*n*=16-30 in each case).
*In wild type, marker genes are expressed in respective NBs always to 100%.
†Position of ocular *ind* domain is exceptional to other *ind* domains; it is unclear whether descending Ppd6,9,13, Pcd7,13 represent intermediate NBs.
Bold indicates marker expression less than 90%.
Bold italics indicate ectopic gene expression.
PCNBs, protocerebral NBs; –, marker gene is not expressed; n.d., not determined; d, dorsal; i, intermediate; v, ventral.

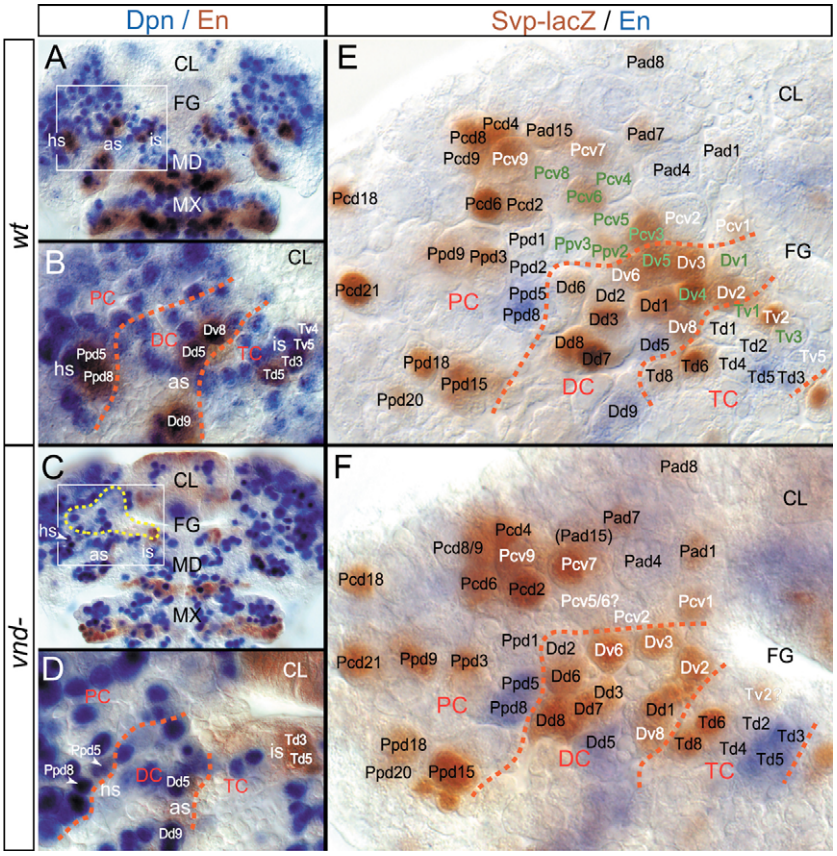

Fig. 3. Defects in brain NB pattern in *vnd* mutants at stage 11. (A-F) Antibody double staining against Engrailed [En; En-*lacZ* (A,B), En-protein (C-F)] and Deadpan (Dpn; A-D) or Seven up-*lacZ* (Svp-lacZ; E,F) in wild type (A,B,E) or in *vnd* mutants (C,D,F). (**A**,**B**) All brain NBs have developed by stage 11. (B) Higher magnification of region framed in A. NBs delaminating from the *en* head spot (hs), the *en* antennal stripe (as) and the *en* intercalary stripe (is) are indicated. (**C**) Broken yellow line encloses ventral pNE, in which NB formation is abnormal. (**D**) Higher magnification of the region framed in C; Dv8, Tv4,5 are missing. (**E**,**F**) All ventral NBs (including the ventral Svp-lacZ-positive) are indicated in white or green, dorsal Svp-lacZ-positive NBs in black inscriptions. (F) In *vnd* mutants, ventral NBs indicated in green in E are missing. Segmental borders between the trito- (TC), deuto- (DC) and protocerebrum (PC) are marked by broken red lines. as, *en* antennal stripe; hs, *en* head spot; is, *en* intercalary stripe; AN, MD, MX, antennal, mandibular, maxillary segment, respectively; CL, clypeolabrum; FG, foregut. Anterior is upwards.

Msh expression in *vnd* embryos is ectopically expanded into the ventralmost pNE of the intercalary and antennal segment and corresponding residual NBs, apparently owing to the lack of repression by *vnd* and *ind* (compare Fig. 6A-D with 6E-H). We observed derepression of Msh already before the early phase of NB formation (stage 8/9; Fig. 6E,F). This is in contrast to observations made in the trunk, where *ind* (instead of *msh*) is derepressed in the ventral neuroectoderm at these stages (McDonald et al., 1998) [although Msh is found in the ventral neuroectoderm at later stages (Chu et al., 1998)]. Early ectopic Msh is likely to 'dorsalize' the ventral pNE and developing residual NBs. We found, that ectopic Msh also encompasses the region of the missing antennal *ind* spot, suggesting that cell fate of intermediate Dd1 (which is mostly formed) may also be affected by ectopic Msh. By contrast, ectopic Msh is largely absent at positions of the intercalary *ind* spot (compare Fig. 6H with 6T), indicating that, at least in the TC, the genetic interaction of *ind* and *msh* (repression of *msh* by *ind*) may be comparable with the situation in the trunk.

Upon ectopic expression of Vnd (*sca-vnd*) in the entire neuroectoderm, we found that Msh, before and during the phase of NB formation, is largely (15%, *n*=28), and sometimes completely, suppressed in the dorsal pNE and descending NBs (compare Fig. 6A-D with 6I-L). In those cases in which residual Msh is found, it is usually confined to dorsalmost ectodermal patches in the head, which, based on their position, are unlikely to give rise to brain NBs.

In *sca-vnd* embryos, expression of *ind* is lacking in the intercalary domain in 85% of cases, and diminished in the ocular domain in 37% cases (*n*=50), indicating that, as in the trunk neuroectoderm, ectopic Vnd is able to suppress *ind* expression (compare Fig. 6M-P with 6U-X). However, the antennal *ind* spot is almost unaffected under these conditions (observed in 92% cases by stage 10/11; *n*=50). Thus, *ind* expression in the antennal (and residual ocular) domain is less sensitive to ectopic Vnd.

Taken together, these data indicate that interactions between *vnd*, *ind* and *msh* differ among brain segments and compared with the VNC (Fig. 6Y). In the brain, *ind* is expressed in three separate segmental domains, and Msh is not expressed in the PC. Whereas in the trunk Vnd negatively regulates *ind*, Vnd does not repress *ind* (but msh) in the ventral pNE and NBs of the DC and TC. Instead, in the deutocerebral region, Vnd is necessary for activating and/or maintaining *ind* expression.

Normal production of glial cells depends on Msh and Vnd in the posterior brain (TC and DC) but not in the anterior brain (PC)

In the VNC, most glial cells derive from dorsal NBs (Schmidt et al., 1997), and *msh* is implicated in proper specification of these NBs and their progeny (Buescher and Chia, 1997; Isshiki et al., 1997). Likewise, Msh is expressed in dorsal pNE and NBs of the TC and DC (Fig. 6A-D). First, glial cells in the TC and DC are closely associated with dorsal NBs, and are thus likely to derive from these progenitors (Urbach et al., 2003) (data not shown). In order to find out whether *msh* influences glial cell development in the brain, we counted Repo-positive cells in *msh* mutant embryos at early stage 12; until this stage it is possible to relate glial cells to their neuromere of origin. We observed that the number of glial cells in the DC and TC is strongly reduced, but is unchanged in the PC (Fig. 7A-D; Table 2).

Conversely, in *vnd* embryos, we observed an increase in the number of glial cells in the DC (>125%, *n*=22) and TC (>30%, *n*=22). In the PC, the number of glial cells seems to be unchanged,

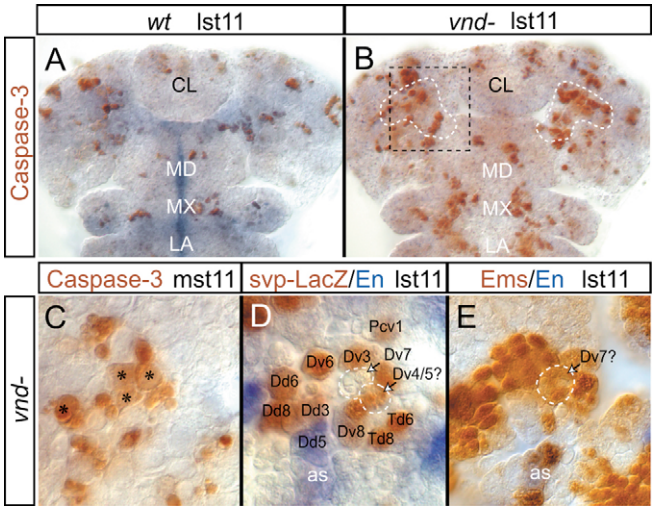

Fig. 4. Increased apoptosis in the *vnd* mutant pNE. (**A**,**B**) Anti-Caspase 3 antibody staining in the head ectoderm at late stage 11 (lst11); blue staining along the midline in A indicates the blue balancer. (A) In heterozygotes (wild type; wt) a few scattered apoptotic cells are observed in the pNE. (B) In *vnd* mutants (*vnd*–), apoptotic cells are significantly more abundant in the pNE, especially in the ventral part (broken white line), and are also more frequent in the ventral neuroectoderm of the mandibular (MD), maxillary (MX) and labial (LA) segment. (**C-E**) NB layer in the DC of *vnd* embryos. A corresponding region is framed by a broken black line in B. (C) Mid-stage 11 (mst11); Caspase-3 is detected in a few ventral deutocerebral NBs (black asterisks). (D,E) Slightly later (lst11), degenerating ventral NBs can be identified at corresponding positions by morphological criteria [e.g. condensation and fragmentation of the nucleus and cytoplasm; Abrams et al. (Abrams et al., 1993)]. (D) Svp-lacZ/En staining suggests that NBs in position of Dv4/5 and Dv7 undergo apoptosis. (E) Ems/En labeling reveals that the degenerating NB (presumably Dv7) ectopically expresses Ems (compare with Fig. 5B). as, *en* antennal stripe; CL, clypeolabrum.

as in *msh* mutants (Fig. 7E,F; Table 2). The supernumerary glial cells in the TC and DC were found at more ventral positions, suggesting that they stem from ventral rather than dorsal brain NBs. This is in accordance with the derepression of Msh in the ventral pNE (described above), which may 'dorsalize' residual ventral NBs. Such misspecified NBs may produce glial cells, as their dorsal counterparts normally do.

In *sca-vnd* embryos, the glial cell numbers in the TC and DC are significantly reduced compared with wild type (>90% of glial cells are missing in the TC and >50% in the DC, n=22), and match exactly with the numbers counted in *msh* mutants (Table 2). Interestingly, in *sca-vnd* embryos, the number of glial cells is also diminished in the PC (Fig. 7G,H; Table 2). This effect cannot be due to Msh repression, as Msh is normally not expressed in the early PC (this study) (Urbach and Technau, 2003c). Instead, specification of dorsal NBs and their glial progeny in the PC must depend on other factors. Although in a *vnd* mutant background these factors (unlike *msh*) do not expand the territory of their glial promoting function, they (like *msh*) remain repressable by ectopic Vnd expression.

Taken together, these data indicate that, in the posterior brain (TC and DC), production of normal numbers of glial cells depends on *msh* expression (as in the VNC), which is ventrally delimited by Vnd (but by Ind in the VNC). This is in clear contrast to the anterior brain (PC), where early glial development is independent from *msh* and *vnd*.

Table 2. Effects of *msh* (loss-of-function) and *vnd* (loss-of-function and gain-of-function) on the number of glial cells

Stage 12	TC	DC	PC
Wild type	3.3±0.5	3.6±1.6	16.2±1.3
msh–	0.1±0.4	1.1±0.4	18.9±2.7
vnd–	4.5±1.4	8.2±1.7	16.4±2.7
sca-vnd	0.2±0.4	1.3±0.9	8.5±1.7

Average number of glial cells in the trito- (TC), deuto- (DC), and proto- (PC) cerebrum in wild type, *msh* mutant (*msh*-), *vnd* mutant (*vnd*-) and *sca-vnd* embryos counted at early stage 12 (12/5) (n=18 for wild type, n=22 under all other conditions).

DISCUSSION

vnd promotes formation of brain NBs

The NK-2 type homeodomain protein Vnd is dynamically expressed in the pNE and the descending brain NBs. In *vnd* mutant embryos, we found at embryonic stages 9 and 11 that ventral NBs in the TC, DC and PC are largely absent, although at different frequencies. Analogously, in the primordium of the VNC of *vnd* embryos a significant loss of ventral NBs has been reported (Jimenez and Campos-Ortega, 1990; Skeath et al., 1994; Chu et al., 1998; McDonald et al., 1998).

In the absence of Vnd, we found an increase in cell death, which contributes to the loss of ventral brain NBs. Apoptosis acts at the level of both pNE progenitor cells and NBs [for cell death occurring at later stages, see Sprecher et al. (Sprecher et al., 2006)]. It is not yet clear whether the reduction of ventral NBs is solely due to cell death, or whether it also involves activity of proneural genes. In the truncal neuroectoderm, proneural genes of the AS-C complex promote NB formation (reviewed by Campos-Ortega, 1995). There is evidence that *vnd* interacts with proneural genes, but also that it has additional function in promoting NB formation apart from activating proneural genes. The latter assumption is supported by the finding that, in *vnd* embryos, *lethal of scute* (*l'sc*) can still be expressed in the ventral proneural clusters of, for example, NB5-2, although the respective NB is missing (Skeath et al., 1994; Chu et al., 1998). In the pNE, genes of the AS-C complex are expressed in large proneural domains, of which those of *achaete*, but especially of *l'sc* (Urbach et al., 2003; Younossi-Hartenstein et al., 1996), seem to overlap with the *vnd* expression domain, suggesting a possible genetic interaction. However, in *vnd* embryos, we observe no substantial differences in the expression pattern of *l'sc* transcript compared with the wild type (R.U., unpublished). Thus, similar to the situation in the trunk, Vnd does not appear to exert proneural function through activation of *l'sc*. However, our data propose a possible interaction between *vnd* and the proneural gene *atonal*. In *vnd* mutants, expression of *atonal* is often missing in proneural clusters of the sensory organ precursors of the hypopharyngeal-latero-hypopharyngeal organ (see Fig. S1 in the supplementary material). Clearly, further investigations are required to clarify in how far interactions between *vnd* and proneural genes play a role in the formation of ventral brain NBs.

We noticed that, in *vnd* mutants, not only ventral, but also intermediate brain NBs in the TC and DC show defects in their formation or specification, comparable with the situation in the trunk (Jimenez and Campos-Ortega, 1990; McDonald et al., 1998; Mellerick and Modica, 2002). As intermediate brain NBs do not express *vnd* (but *ind*), these defects appear to be non-cell-autonomous. Another, more likely explanation is that determination occurs at the blastodermal stage, when Vnd is transiently expressed in a much larger population of cells in the pNE, which presumably

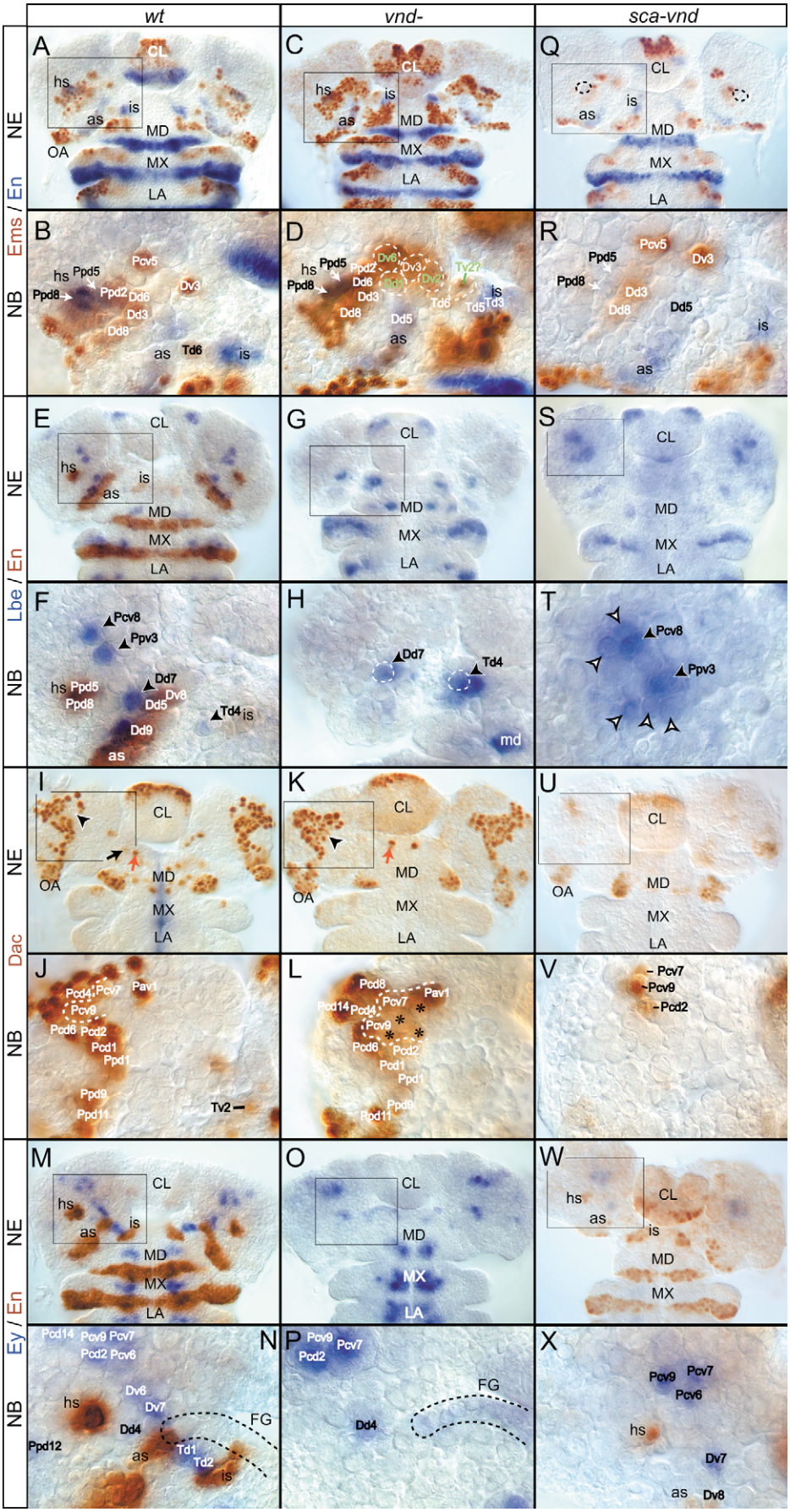

Fig. 5. See next page for legend.

Fig. 5. Defects in the brain NB pattern in *vnd* loss-of-function and gain-of-function embryos at stage 11, as revealed by marker genes specific for NB subsets. (A-X) Head flat preparations antibody labeled against Engrailed [En; En-lacZ (E,F,M,N) or En-protein (A-D,Q,R,W,X)] and Empty spiracles (Ems; A-D,Q,R) or Ladybird early (Lbe; E,F) or Eyeless (Ey; M,N,W,X), only Ey (O;P), Lbe (G,H,S,T) and Dachshund (Dac; I-L,U,V) in wild type (*wt*), *vnd* mutant (*vnd-*) and *sca-vnd* embryos as indicated; ventral views (anterior is upwards). (B,D,F,H,J,L,N,P,R,T,V,X) Higher magnifications at the level of NBs deriving from neuroectodermal regions (NE) framed in A,C,E,G,I,K,M,O,Q,S,U,W. (**A**,**B**) Ems is expressed in dorsal NBs in the PC (Ppd2,5,8; the latter two co-express En), DC (Dd3,6,8) and TC (Td6), and in the ventral Pcv5 and Dv3. (**C**) In *vnd* embryos, Ems is derepressed in the ventral trito-/deutocerebral NE and (**D**) in residual ventral/intermediate NBs (indicated with green inscriptions). Dorsal NBs are all identifiable; the ventral Pcv5 is missing. (**E**) In wild type, Lbe is downregulated in the pNE, (**F**) confined to the dorsal Td4 and Dd7, and to the ventral Pcv8 and Ppv3. (**G**) In *vnd–*, the Lbe domain in the TC and DC is ventrally slightly enlarged. (**H**) Conversely, Pcv8 and Ppv3 are missing. (**I**,**J**) In heterozygotes, Dac is found in about 10 dorsal and three ventral NBs (border indicated by the broken white line in J). Black arrow in I indicates Dac-positive Tv2, red arrow indicates an adjacent Dac cell in the MD. Blue midline staining in I indicates blue balancer. (**K**,**L**) In *vnd–* Dac is ectopically expressed in part of the ventral protocerebral pNE (black arrowhead in K) and approximately four descending NBs (black asterisks in L). Dac expression is absent in position of Tv2, and reduced in the MD. (**M**,**N**) Ey-positive NBs are indicated in the TC, DC and PC of wild type. (**O**,**P**) In *vnd–*, Ey is missing at positions of ventral (Dv6, Dv7, Pcv6) and intermediate (Td1, Td2) NBs, but is found in all dorsal NBs (e.g. Dd4 and Pcd2). There is faint ectopic Ey in the ventral intercalary NE (encircled by broken black line in P; the corresponding area is encircled in N). Similarly, ectopic Ey is expressed in ventral NE of the mandibular (MD), maxillary, (MX) and labial (LA) segments (O). (**Q**) In *sca-vnd*, Ems is largely repressed in the dorsal pNE of the TC, DC and PC, but not in the ventral pNE. In addition, En is absent from the *en hs* (corresponding region encircled with a broken line), and diminished in the *en* antennal stripe (as). (**R**) The ventral Pcv5 and Dv3 express Ems. The dorsal Dd5 lack En; Ppd5 and Ppd8 lack En and Ems. (**S**) Lbe is absent in the TC and DC but expanded in the PC. (**T**) Ppv3 and Pcv8, close to the border of the dorsal PC, express Lbe the strongest; additionally, about five dorsal NBs express ectopic Lbe (white arrowheads). (**U**) Dac is repressed in the ocular pNE and (**V**) in dorsal protocerebral NBs, but retained in ventral NBs (Pcv7,9). (**W**) Ey is repressed in the dorsal pNE of the DC and PC, and is absent in the TC, MD, MX and LA. (**X**) Ey expression is lacking in dorsal, but retained in most ventral, NBs in the PC and DC. CL, clypeolabrum; FG, foregut; OA, optic lobe anlagen.

include progenitors of intermediate NBs. A similar proposal was made for intermediate NBs in the trunk (McDonald et al., 1998). Furthermore, early commitment of ventral neuroectodermal cells and cell-autonomous expression of ventral and intermediate NB fates has been demonstrated by heterotopic transplantations of neuroectodermal cells from ventral to dorsal sites at the early gastrula stage (Udolph et al., 1995).

vnd is essential for specification of ventral and repression of dorsal fate in brain NBs

In the trunk, a segmentally reiterated combinatorial code of genes expressed within each particular proneural cluster specifies the individual identity of the NB it gives rise to. These include DV patterning genes and segment polarity genes, which provide positional information in the neuroectoderm, as well as a number of other factors (reviewed by Bhat, 1999; Skeath, 1999; Skeath and Thor, 2003). Most of these genes are also expressed in specific domains of the pNE before NBs delaminate, although in a segment-specific manner (Urbach and Technau, 2003b). Our present data show that Vnd influences the expression of such site-specific marker genes ('NB identity genes') already in the pNE, before NBs are formed. In *vnd* embryos, we noticed a derepression of dorsal-specific genes in the ventral pNE (e.g. of *msh* and *ems* in the intercalary and antennal segment, and *dac* in the ocular segment) and in the descending NBs, and conversely, a loss of ventral-specific gene expression (e.g. *lbe* in the PC). The altered expression of 'NB identity genes' in *vnd* mutants reflects a ventral-to-dorsal transformation of ventral pNE and residual NBs. Further evidence for such a transformation is the production of (ectopic) glial cells by these ventral NBs, which normally is a trait specific to dorsal NBs (see below). By contrast, in the trunk, absence of Vnd results in a ventral-to-intermediate transformation, owing to the derepression of *ind* (instead of *msh* in pNE), which induces specification of intermediate NB fates (Chu et al., 1998; Weiss et al., 1998).

Together, our data in the *vnd* loss- and gain-of-function backgrounds indicate that *vnd* is required, and is at least partially sufficient, for the induction of ventral fate in brain NBs through the activation of genes specific for the ventral pNE, and through the repression of genes specific for dorsal pNE.

Interactions between columnar genes *vnd, msh* and *ind* differ in the neuroectoderm of head and trunk

Our analysis revealed differences in the regulation of DV patterning genes among the intercalary (IC), antennal (AN) and ocular (OC) head segments, giving rise to the TC, DC and PC, respectively. Overexpression of *vnd* leads to repression of *ind* within the IC, but loss of *vnd*-function does not seem to cause ventral expansion of the *ind* intercalary spot. Unexpectedly, *ind* is completely absent in the AN of *vnd* mutants, suggesting that in this segment *vnd* is necessary for activation and/or maintenance of *ind* (rather than repression). This is supported by our previous finding that the *ind* antennal spot transiently co-expresses Vnd (Urbach and Technau, 2003c), which is unique in the neuroectoderm, and by our present finding that in *vnd* gain-of-function background the *ind* antennal spot is almost unaffected. In the OC, however, *ind* expression is partially repressed upon Vnd overexpression, and ventrally expanded in the absence of Vnd, similar to the situation in truncal segments. However, because, in wild type the *ind* ocular spot does not adjoin the ocular *vnd* domain (Urbach and Technau, 2003c), its expansion in *vnd* embryos cannot be due to a cell-autonomous effect.

Overexpression of *vnd* abolishes Msh almost completely in the neuroectoderm of all body segments. Yet, absence of Vnd reveals segment-specific differences in the regulation of *msh*. Owing to insulated *ind* expression in the IC and lack of *ind* in the AN of *vnd* mutants, Msh (instead of *ind*) is found in the ventral pNE of these segments, which is unique in the CNS anlagen, except for the mandibular segment, which exhibits equivalent expression (compare Fig. 6A,M with 6E,Q).

Among the pregnathal segments, the degree of conservation with regard to the expression and interactions of DV patterning genes seems to be highest in the posterior IC (TC) [*ind* and *msh* being repressed by (ectopic) *vnd*, and *msh* by *ind*]. In the anterior head, endogenous Msh expression in the dorsal pNE reaches the segmental border between AN (DC) and OC (PC), but does not cross

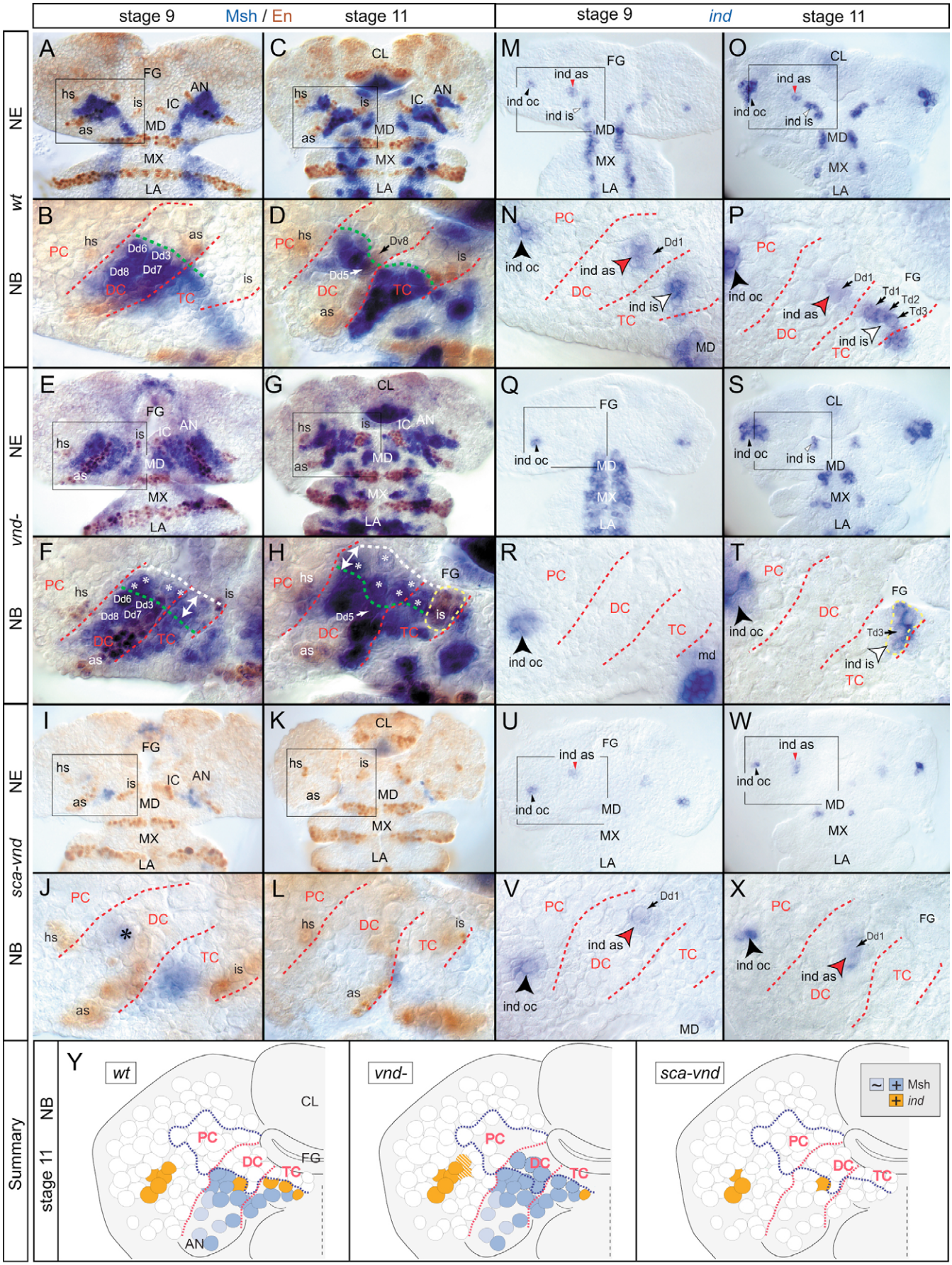

Fig. 6. See next page for legend.

Fig. 6. *vnd* regulates expression of *ind* and *msh* in the pNE and brain NBs. Head flat preparations double stained with antibodies against Msh and En (A-L), or subjected to in situ hybridization against *ind* (M-X) of wild-type (*wt*; A-D,M-P), *vnd* mutant (*vnd-*; E-H,Q-T) and *sca-vnd* embryos (I-L,U-X); ventral views; stages as indicated. (B,D,F,H,J,L,N,P,R,T,V,X) Higher magnifications of the regions framed in A,C,E,G,I,K,M,O,Q,S,U,W, focusing on NBs and neuroectoderm (NE), respectively. (**A**,**C**) In wild type, Msh is found in the dorsal NE of the intercalary (IC), antennal (AN) and gnathal segments (MD, mandibular, MX, maxillary, LA, labial). (**B**,**D**) Dorsal NBs in the DC (B), and later in the TC/DC (D), express Msh. The ventral border of the Msh domain is indicated by a green broken line. It runs between the En-positive Dd5 and Dv8 (which does not express Msh). (**E**,**G**) In *vnd–*, Msh is expanded into the ventral pNE of the IC and DC. Ectopic Msh is not found in the PC. (**F**,**H**) Residual ventral NBs (white asterisks) in the TC and DC express ectopic Msh. Broken green line indicates the ventral border of the wild-type Msh domain (compare with B,D); the broken white line indicates ectopic Msh expression. (H) Ectopic Msh is found at lower levels in the *en* intercalary stripe (is; encircled with broken yellow line) owing to repression by *ind,* which is partly co-expressed (encircled with a broken yellow line in T). (**I**,**K**) In *sca-vnd*, Msh is largely repressed in the dorsal pNE. Low levels of Msh can be found in dorsalmost ectodermal patches in the head and trunk, and (**J**) sometimes in a dorsal NB in the DC (black asterisk). (**L**) Msh is almost completely repressed in NBs. (**M**,**O**) *ind* is expressed in three spots in the pNE of the intercalary (*ind is*, white arrowhead), antennal (*ind as*, red arrowhead) and ocular (*ind oc*, black arrowhead) segments. (**N**) The *ind as* gives rise to Dd1. (**P**) The *ind is* gives rise to Td1, Td2 and Td3. (**Q**,**R**) In *vnd* mutants, *ind is* and *ind as* are absent. *ind* is derepressed in ventral NE of the MD, MX and LA. (**S**,**T**) *ind oc* appears ventrally expanded; *ind is* becomes identifiable, but its overall size is reduced; *ind as* remains absent. (**U**,**W**) In *sca-vnd*, *ind* is largely repressed in the pNE. (**V**,**X**) Size of *ind oc* is diminished; *ind is* is absent, but *ind as* (and Dd1) is unaffected. (**Y**) Schemes summarizing the expression of *ind* and Msh in NBs of wild-type, *vnd* mutant and *sca-vnd* embryos at stage 11. Broken blue line encloses ventral NBs that, in wild type, persistently or transiently express Vnd until stage 11. Segmental borders are marked by broken red lines. as, *en* antennal stripe; hs, *en* head spot; is, *en* intercalary stripe; AN, IC, MD, MX, LA, antennal, intercalary, mandibular, maxillary, labial segment, respectively; CL, clypeolabrum; FG, foregut. Anterior is upwards.

it (Urbach and Technau, 2003c). Ectopic Msh in *vnd* mutants does also not cross this border, which suggests interference with regulatory factors acting in AP axis.

Significant differences between the anterior head segments and the trunk have also been reported for the initial mode of activation and cross-regulatory interactions of segment-polarity genes (Gallitano-Mendel and Finkelstein, 1997).

Requirement for further factors in DV patterning of the anterior brain

As discussed above, in the pNE, *vnd* is necessary for the formation and specification of brain NB. It remains to be shown whether *ind* and *msh* exert analogous functions. However, more than 50% of the identified brain NBs do not express any of the three DV patterning genes (Urbach and Technau, 2003c). Most of these NBs derive from pNE of the preantennal head, which implies that further factors are involved in DV patterning of the anterior pNE and brain. Several other genes have been reported to be crucial for DV patterning in the truncal neuroectoderm, such as the EGF-receptor homolog *Egfr* (Skeath, 1998; Udolph et al., 1998; Yagi et al., 1998), the Sox genes *SoxNeuro* and *Dicheate* (Buescher et al., 2002; Sanchez-Soriano and Russell, 2000; Zhao and Skeath, 2002), and *Nk6* (Uhler et al., 2002). For most of them it has been shown that they are involved in formation and/or specification of truncal NBs. *Egfr*, both Sox genes (J.S. and R.U., unpublished) and *Nk6* (Uhler et al., 2002) are also expressed in the pNE, before and during the phase of NB formation. However, in *Egfr* mutant embryos the number and pattern of brain NBs is unaffected (Dumstrei et al., 1998). How far the Sox genes and *Nk6* contribute to the formation and/or specification of brain NBs awaits further investigation.

Segment-specific requirements of Msh and Vnd for the production of glial cells in the brain

Most of the glial cells in the VNC derive from dorsal NBs (neuroglioblasts or glioblasts) (Schmidt et al., 1997), which depend on *msh* for proper specification. Accordingly, glial cells deriving from these progenitors are missing or improperly differentiated in *msh* mutants (Isshiki et al., 1997), as well as in *sca-vnd* embryos (Mellerick and Modica, 2002). Likewise, in the TC and DC, first glial cells are closely associated with dorsal NBs that descend from Msh-expressing pNE (Urbach and Technau, 2003c). In the TC, some dorsal NBs have been identified as glial progenitors, e.g. the neuroglioblast Td4 and the glioblast Td7, which are putative serial homologs of the truncal neuroglioblast NB5-6 and the glioblast LGB, respectively (Urbach et al., 2003). In absence of Vnd, we found the number of glial cells in the TC, and especially in the DC, to be increased. This is most probably due to the segment-specific early derepression of Msh in the ventral pNE and NBs of the TC and DC. In the truncal segments, however, *ind* instead of Msh is derepressed in the ventral NE (Chu et al., 1998; McDonald et al., 1998), and the number of glial cells is not significantly affected in the VNC of *vnd* mutants (data not shown). Furthermore, in *msh* mutants, glial development in the TC and DC is almost completely abolished, which parallels the phenotype observed upon *vnd* overexpression (leading to repression of *msh* in the dorsal pNE and NBs). Thus, comparable with the situation in the VNC, Msh promotes glial fate in the TC and DC. However, in the PC, glial development must be regulated differently (at least in its early phase). Until stage 12 we did not detect Msh in this part of the brain, and in *msh* mutants the number of glial cells in the PC is normal. Glial cell fate in the PC is also not affected by loss of *vnd*, although it remains repressable by ectopic Vnd. Therefore, as opposed to the TC and DC, and to the VNC, normal production of early glial cells in the PC does not depend on *msh*, nor indirectly on *vnd* or *ind*.

Evolutionary conservation of DV patterning in the brain

There are striking similarities in the spatial order of expression of *vnd*, *ind* and *msh* in the *Drosophila* neuroectoderm and homologous genes in the neural plate and neural tube of vertebrates: *vnd* homologs of the *Nkx2* family are expressed in ventral regions; the *ind* homologs *Gsh1* and *Gsh2* are expressed in the intermediate regions; the *msh* homologs *Msx1*, *Msx2* and *Msx3* are expressed in the dorsal region of the neural tube (reviewed by Arendt and Nübler-Jung, 1999; Cornell and Ohlen, 2000). This dorsoventral order of expression is conserved not only in the anlagen of the truncal CNS but also in those that form the posterior part of the brain (in *Drosophila*, TC and DC; in vertebrates, hindbrain) (Urbach and Technau, 2003c). Moreover, the anterior borders of the expression

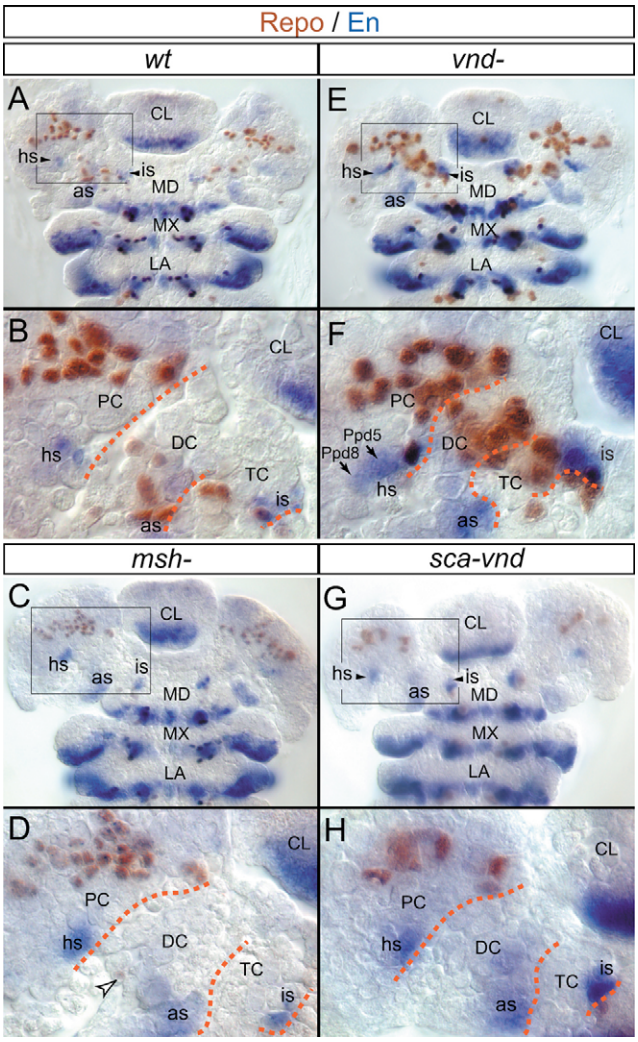

Fig. 7. Vnd represses glial fate in the early trito- and deutocerebrum. (A-H) Head flat preparations double stained with antibodies against the glial marker Repo and En in wild-type (*wt*; A,B), *msh* mutant (*msh–*; C,D), *vnd* mutant (*vnd–*; E,F) and *sca-vnd* embryos (G,H) at early stage 12, all in a ventral view. (B,D,F,H) Close-ups of regions framed in (A,C,E,G), respectively. (**A**,**B**) In the TC and DC, first glial cells develop in the dorsal pNE. In contrast, in the PC, some of the glial cells appear to develop from ventral NBs. Broken red lines indicate segmental boundaries. (**C**,**D**) In *msh–*, glial cells in the TC and DC are almost absent (one cryptic glial cell is detected at dorsalmost position in the DC; white arrowhead in D). Similarly, the number of glia cells in the MD, MX and LA is diminished (C). Glial cells are not affected in the PC. (**E**,**F**) In *vnd–*, the number of glial cells is significantly increased in the TC and DC. Most of the ectopic glial cells develop in the ventral TC and DC (F), as is indicated by their position relative to the *en* antennal stripe (*as*) and *en* head spot (*hs*). (**G**,**H**) In *sca-vnd*, glial cells are absent in the TC and DC, strongly resembling the phenotype in *msh–*. However, glia cell number is also reduced in the PC.

domains of these columnar genes correspond in the early brains of *Drosophila* and mouse: expression of *vnd/Nkx2* extends most rostrally (mouse ventral forebrain), followed by *ind/Gsh1* and, finally, *msh/Msx3* expression (reviewed by Urbach and Technau, 2004). Thus, the expression of columnar genes in the brain is, to some extent, evolutionarily conserved both along the DV axis and along the AP axis.

In this study, we have presented evidence that in *Drosophila vnd* mutant embryos a large fraction of ventral brain NBs is missing, and that ventral pNE and residual ventral NBs show significant traits of a ventral-to-dorsal transformation owing to derepression of *msh* (as opposed to *ind* in the VNC). Again, this displays obvious similarities to findings made in mice carrying a deletion of *Nkx2.1*. Consistent with the pattern of expression in wild type, in the mutant embryonic brain a substantial loss of ventral (especially forebrain) structures has been observed (Kimura et al., 1996; Sussel et al., 1999). Moreover, *ind/Gsh2* expression is not expanded in Nkx2.1 mutants (Corbin et al., 2003) (reviewed by Rallu et al., 2002), and residual basal (ventral) pallidal structures become transformed into dorsal striatal structures (Sussel et al., 1999). Thus, in both *Drosophila* and mouse, loss of *vnd/Nkx2* in the brain leads to a transformation of ventral into dorsal structures, rather than into intermediate structures, which has been shown to be the case in the truncal CNS of both species (Briscoe et al., 1999; Chu et al., 1998; McDonald et al., 1998). Therefore, in the developing brains of *Drosophila* and vertebrates, *vnd/Nkx2* is crucial for the formation and specification of ventral brain structures, and interacts with other dorsoventral patterning genes in a region-specific manner.

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Supplementary material

Supplementary material for this article is available at
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