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Differential effects of EGF receptor signalling on neuroblast lineages along the dorsoventral axis of the *Drosophila* CNS

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SUMMARY

The *Drosophila* ventral nerve cord derives from a stereotype population of about 30 neural stem cells, the neuroblasts, per hemineuromere. Previous experiments provided indications for inductive signals at ventral sites of the neuroectoderm that confer neuroblast identities. Using cell lineage analysis, molecular markers and cell transplantation, we show here that EGF receptor signalling plays an instructive role in CNS patterning and exerts differential effects on dorsoventral subpopulations of neuroblasts. The *Drosophila* EGF receptor (DER) is capable of cell autonomously specifiying medial and intermediate neuroblast cell fates. DER signalling appears to be most critical for proper development of intermediate neuroblasts and less important for medial neuroblasts. It is not required for lateral neuroblast lineages or for cells to adopt CNS midline cell fate. Thus, dorsoventral patterning of the CNS involves both DER-dependent and -independent regulatory pathways. Furthermore, we discuss the possibility that different phases of DER activation exist during neuroectodermal patterning with an early phase independent of midline-derived signals.

Key words: *Drosophila*, CNS, Cell fate, Neuroblast, EGF receptor signalling, Dorsoventral patterning

INTRODUCTION

The development of the central nervous system (CNS) involves the transformation of a uniform epithelial sheet, called neuroectoderm, into a highly complex organ comprising a vast number of different neuronal and glial cell types each of which expresses specific structural and functional characteristics. The mechanisms that lead to this cellular diversity in the CNS are largely unknown. The *Drosophila* embryo is a well-suited model system to approach such mechanisms. The embryonic ventral nerve cord of *Drosophila* is relatively simple, comprising about 800 cells per neuromere. In a first step of neurogenesis, the neurogenic region of the ectoderm is determined by the products of early patterning genes (e.g. St Johnston and Nüsslein-Volhard, 1992). Within the neuroectoderm, cells are then selected that in the second step will delaminate as CNS progenitor cells, the so-called neuroblasts (NBs). In each hemisegment, about 30 NBs delaminate according to a well-defined spatial and temporal pattern (Hartenstein and Campos-Ortega, 1984; Doe, 1992; Broadus et al., 1995). This step is under the control of two sets of genes, the proneural genes and the neurogenic genes (e.g. Campos-Ortega, 1993). A second set of about 8 CNS progenitors per segment derives from one row of mesectodermal cells on either side of the ventral midline which behave differently from the NBs in many respects. The entire embryonic lineages of the NBs and the CNS midline progenitors have been recently described (Bossing and Technau, 1994, Bossing et al., 1996; Schmidt et al., 1997). Each of the NBs gives rise to a characteristic cell lineage consisting of specific neuronal and/or glial cell types. Furthermore, each of them expresses a specific set of molecular markers (Doe, 1992; Broadus et al., 1995). However, the mechanisms specifying the individual NB identities are not yet clarified.

The position of each NB in the subectodermal NB layer reflects its position of origin within the neuroectoderm (e.g. Doe, 1992; Bossing et al., 1996). Furthermore, cell transplantation experiments provide indications that NBs at least partially acquire their identities in the neuroectoderm before delamination (Prokop and Technau, 1994; Udolph et al., 1995). This suggests that positional information in the neuroectoderm plays an essential role in NB specification. Indeed, for the anterior-posterior axis, the products of segmentation genes appear to play a pivotal role in providing this information (e.g. Martin-Bermudo et al., 1991; Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Bhat, 1996; Matsuzaki and Saigo, 1996).

Much less is known about the factors involved in the specification of NBs along the dorsoventral axis. In response to the amount of nuclear DORSAL protein, early patterning genes are differentially expressed leading to the determination of distinct dorsoventral anlagen, one of which is the neuroectoderm (Rushlow et al., 1989; St Johnston and Nüsslein-Volhard, 1992; Steward and Govind, 1993; Morisato and Anderson, 1995). Within the neuroectoderm, a heterogeneity in the state of cell

determination has been detected depending on dorsoventral position. Furthermore, inductive signals have been shown to exist at ventral sites of the neuroectoderm to confer ventral NB identities (Udolph et al., 1995). A good candidate to confer such signals is the *Drosophila* EGF receptor (DER) signalling pathway. DER is a transmembrane protein and belongs to the class of receptor tyrosine kinases (RTK). RTKs play important roles in many developmental decisions and cellular interactions (reviewed in Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). In *Drosophila*, it has been found that DER is required for proper patterning of another neuroectoderm-derived tissue, the ventral epidermis (Raz and Shilo, 1993; Schweitzer et al., 1995a), and for patterning and induction of cells in the ventral mesoderm (Lüer et al., 1997; Zhou et al., 1997). DER is thought to be differentially activated in the ventral neuroectoderm by the secreted SPITZ protein and the ventral midline serves as a local source for this ligand (Golembo et al., 1996b; Schweitzer and Shilo, 1997).

Although the impact of DER signalling on ventral epidermal and mesodermal tissues has been demonstrated, it remains to be clarified whether DER is also involved in the specification or differentiation of CNS lineages. Here we show on the level of identified lineages that DER signalling influences NB development and exerts differential effects on dorsoventral subpopulations of NBs. In particular, the development of intermediate NBs critically depends on DER, whereas the medial NBs are less DER dependent. DER is dispensable for lateral NB lineages and it is also not required for cells to adopt CNS midline fate. These differential effects point to the existence of additional factors acting independent of and in parallel to DER signalling. Evidence suggesting that DER activation during neuroectoderm patterning does not solely depend on midline-derived signals is discussed.

MATERIALS AND METHODS

Fly strains

Oregon R was used as wild-type strain. *faint-little-ball* mutant flies (flb^{IK35}) carrying a null allele of the *DER* gene (Schejter and Shilo, 1989) were obtained from the Tübingen Stock-Center. The flb^{IK35} stock was rebalanced over a *CyO-ftz* blue chromosome and used throughout the experiments. Standard methods were used to rear flies and collect embryos.

Cell transplantations

Cell transplantations were performed as described in Prokop and Technau (1993). Donor embryos were labelled by injecting a mixture of 3% Fluorescein-isothiocyanate-dextran (FITC-dextran, Sigma) and 3% Horseradish-peroxidase (HRP, Boehringer Mannheim). Transplantation of single cells was monitored using an inverted fluorescence microscope. Cells were taken from different dorsoventral positions of donors (flb^{IK35} mutant, wild-type or heterozygous embryos) at the early gastrula stage (stage 7; stages are described in Campos-Ortega and Hartenstein, 1997) and were isotopically or heterotopically implanted into wild-type hosts at the same stage (see Fig. 1). Host embryos were allowed to develop until stage 16/17 and were further processed according to Prokop and Technau (1993).

Histological techniques

Mutant donors were distinguished from wild type by lack of the blue balancer. Individual donors were stained for β-galactosidase expression by incubation in standard X-Gal staining solution (e.g. Prokop and Technau, 1993) for at least 2 hours at 37°C. Clones obtained from *flb/CyO* and *CyO/CyO* donor cells showed no obvious abnormalities compared to those obtained from wild-type cells.

Antibody stainings were performed as described in Schmidt-Ott and Technau (1992). The following primary antibodies were used: rabbit anti-EVEN-SKIPPED (M. Frasch), anti-REPO (Halter et al., 1995), anti-EAGLE (Dittrich et al., 1997) and mouse monoclonals anti-ENGRAILED/INVECTED (mAb 4D9; DSHB Hybridoma Center, Iowa), and anti-LADYBIRD early (mAb 1D6; Jagla et al., 1997).

Preparations were documented using an Axiophot (Zeiss) microscope equipped with a digital camera (Kontron Progress 3012). Different focal planes were combined using Photoshop (Adobe).

RESULTS

As previously shown by heterotopic transplantation experiments, the extent of determination differs among cells along the dorsoventral axis of the early gastrula neuroectoderm (NE). Whereas cells of the dorsal part of the NE, which normally produce lateral neuroblast (NB) lineages, can be induced at ventral sites to take over the fate of medial and intermediate NBs and of CNS midline progenitors, cells from the ventral part of the NE or from the midline retain their fate upon heterotopic transplantation into the dorsal NE (Udolph et al., 1995). This indicated the existence of autonomous ventral properties and non-autonomous ventralizing signals. As outlined above, the DER signalling pathway is a good candidate to be involved in confering these signals. We tested this hypothesis using cell lineage analysis, molecular markers and cell transplantation to trace the fate of DER-deficient NBs that derive from different dorsoventral regions of the NE.

DER is cell autonomously required for the formation of intermediate NB lineages

The delamination of the NBs from the NE occurs between embryonic stages 8 and 11 (staging according to Campos-Ortega and Hartenstein, 1997) and is divided into five phases (S1-S5). S1-S3 NBs form three longitudinal columns in the NB-layer: medial, intermediate and lateral (Hartenstein and Campos-Ortega, 1984). S4 and S5 NBs become interspersed between the existing columns of NBs (Doe, 1992). The neuroectodermal sites of origin have been traced for the individual NBs and it has been shown that the mediolateral arrangement of the NBs in the NB-layer correponds to the ventrodorsal sites of their delamination from the NE (Bossing et al., 1996; Schmidt et al., 1997). In order to address the various NBs and their lineages with regard to their dorsoventral distribution, they are assigned to the following categories (see NB-maps of Broadus et al., 1995 and Schmidt et al., 1997): medial NBs: 1-1, 2-1, 2-2, 3-1, MP2, 4-1, 5-1, 5-2, 6-1, 7-1; intermediate NBs: 1-2, 2-3, 3-2, 4-2, 4-3, 5-3, 5-4, 6-2, 7-2, 7-3; lateral NBs: 1-3, GP, 2-4, 2-5, 3-3, 3-4, 3-5, 4-4, 5-5, 5-6, 6-4, 7-4, y.

Using the cell transplantation technique, we first tested whether DER might be autonomously required for the specification and development of the NB lineages per se. We analyzed the composition of complete lineages produced by single *faint-little-ball* (*flb*) mutant progenitors in a wild-type background. Early gastrula embryos (stage 7) of the flb^{IK35} mutant strain, which carries a null allele of DER (Shejter and Shilo, 1989), were used as donors.

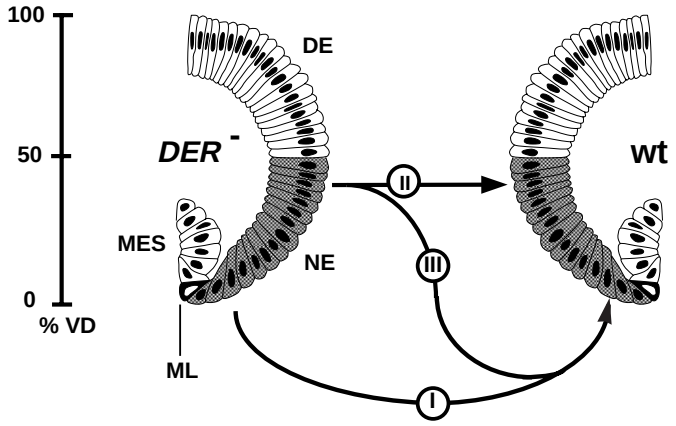

Fig. 1. Diagram of transplantation experiments. Half cross-sections through the early gastrula embryo (stage 7). The neuroectoderm (NE, shaded) represents the ventral half of the ectoderm (0-50% VD). Cells were transplanted from different dorsoventral NE positions of *DER* mutant (*DER*$^{-}$) or wild-type donors (left) into isotopic (I, II) or heterotopic positions (III) of wild-type (wt) hosts (arrows). DE, dorsal ectoderm; MES, mesoderm; ML, midline; NE, neuroectoderm; % VD, % ventrodorsal diameter.

The NE in the truncal region of the early gastrula embryo spans between 0% and about 50% of the ventrodorsal diameter (VD; Technau and Campos-Ortega, 1985). In one set of experiments, we took cells from the ventral part of the NE (vNE, 0-20% VD) of *flb*^{IK35} donors and transplanted them isotopically into wild-type hosts at the same stage (I in Fig. 1). 0-20% VD corresponds to 0-25% of the three-dimensional ventrodorsal perimeter of the embryo (Prokop and Technau, 1993). This region of the NE normally gives rise to medial and intermediate NB lineages (Bossing et al., 1996). In the wild-type controls, we did not obtain all possible types of these lineages and the frequencies among the ones obtained are very uneven (I in Table 1; WT→WT). One reason for this is the limited number of transplantations performed. Furthermore, a fraction of the intermediate NB lineages derives from the region between 20% and 30% VD, which we excluded from the transplantations to avoid overlap between the vNE and dNE domains considered in the various experiments. Thus, our data do not allow us to draw conclusions on each particular type of NB-lineage. However, as outlined above, the dorsoventral sites of origin in the NE are known for all the lineages and each clone obtained can be assigned to one of the previously defined regional subclasses (medial, intermediate and lateral NBs). Therefore, in the

Table 1. NB and midline lineages derived from isotopically and heterotopically transplanted *DER* mutant and wild-type cells

	Transplantations (stage 7) % VD	Types of lineages (stage 17)			
		ML	mNB	iNB	lNB
I.	*DER* 0-20 → WT 0-20	*n* = 24 VUM (10), MP1 (7), MNB (1), UMI (1), ML? (5)	*n* = 21 (60%) MP2 (8), 1-1 (8), 4-1 (1), 5-1 (1), 5-2 (2), 7-1 (1)	*n* = 5 (14%) 1-2 (5)	*n* = 9 (26%) 2-4 (2), 2-5 (4), 3-5 (2), 6-4 (1)
	WT 0-20 → WT 0-20 (a)	*n* = 30 VUM (11), MP1 (8), MNB (5), UMI (3), ML? (3)	*n* = 82 (60%) MP2 (35), 1-1 (16), 2-1 (3), 4-1 (6), 5-1 (2), 5-2 (9), 6-1 (2), 7-1 (9)	*n* = 54 (40%) 1-2 (27), 3-2 (16), 4-2 (6), 6-2 (1), 7-2 (4)	–
II.	*DER* 30-50 → WT 30-50	–	–	–	*n* = 7 1-3 (2), 2-4 (1), 5-6 (1), 6-4 (1), 7-4 (2)
	WT 30-50 → WT 30-50 (b)	–	–	–	*n* = 6 1-3 (1), clone y (2) 2-4 (1), 6-4 (1), 7-4 (1)
III.	*DER* 30-50 → WT 0-20	*n* = 11 MP1 (6), MG (2), ML? (3)	–	*n* = 1 (4%) 7-3 (1)	*n* = 22 (96%) 1-3 (1), 2-4 (4), 2-5 (6), 3-4 (1), 3-5 (1), 7-4 (5), GP (4)
	WT 30-50 → WT 0-20 (c)	*n* = 18 VUM (2), MP1 (9), MNB (1), UMI (1), MG (3), ML? (2)	*n* = 11 (21%) MP2 (2), 1-1 (1), 2-2 (3), 3-1 (1), 4-1 (2), 5-1 (1), 7-1 (1)	*n* = 41 (79%) 1-2 (6), 3-2 (9), 4-2 (12), 6-2 (1), 7-2 (12), 7-3 (1)	–

I - III, transplantation experiments (compare Fig. 1); regions of cell removal from *DER* mutant or wild-type (WT) donors and implantation into wild-type hosts are indicated in % VD. ML, midline lineages; mNB, median NB lineages; iNB, intermediate NB lineages; lNB, lateral NB lineages; *n*, number of identified clones for each category (% refer to total number of NB lineages); individual types of lineages are indicated with the number of cases shown in brackets; ML?, midline clones that could not be assigned to a particular type.

(a) see also Bossing et al. (1996).

(b) see also Udolph et al. (1995) and Schmidt et al. (1997).

(c) see also Udolph et al. (1995).

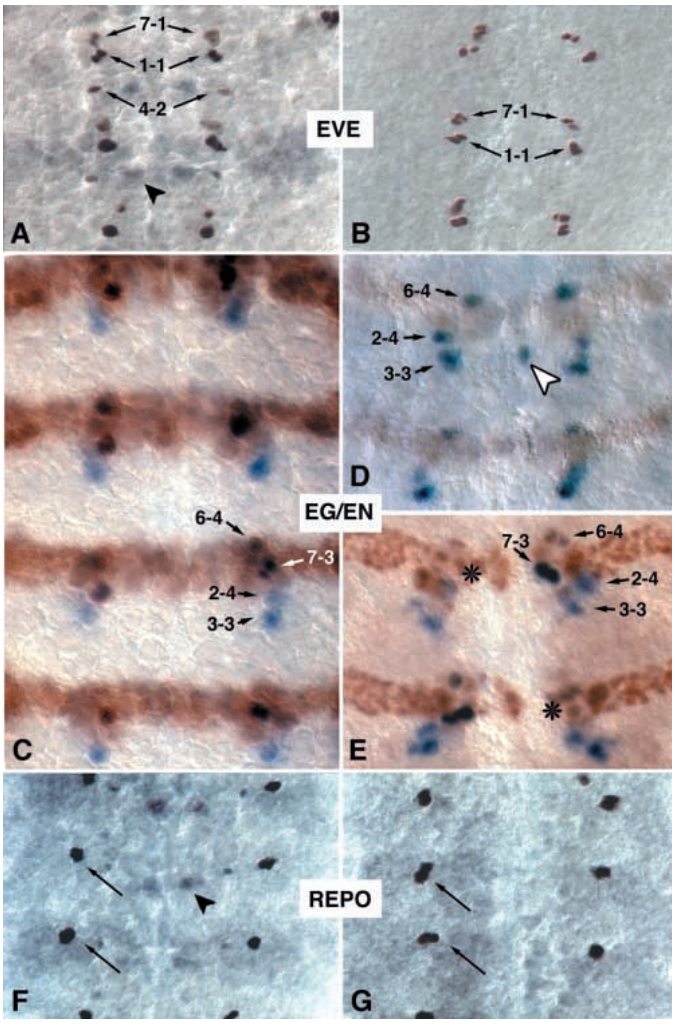

Fig. 2. DER affects the expression of cell-specific markers. Antibody stainings of wild-type (A,C,F) and *DER* mutant (B,D,E,G) embryos. Horizontal views of flat preparations (anterior to the top). (A,B) Anti-EVEN-SKIPPED (EVE) staining; stage 11. (A) In wild-type embryos, the aCC/pCC and the CQ and fpCC neurons express EVE, which represent progeny of NB 1-1 and NB 7-1, respectively (arrows). The first GMC of NB 4-2, GMC 4-2a, is also stained. The arrowhead points to the MP2-expressing *ftz-lacZ* indicative for the blue-balancer (see Materials and Methods). (B) In *DER* mutant embryos, EVE-positive progeny of NB 1-1 and NB 7-1 are formed, whereas the EVE-positive GMC 4-2a is missing. (C-E) Anti-EAGLE (EG)/anti-ENGRAILED (EN) double staining; early (D) and late (C,E) stage 11. (C) In wild-type, the lineages of NB 2-4 and NB 3-3 express EG (blue) whereas those of NB 6-4 and NB 7-3 are positive for EG and EN (black). (D) In *DER* mutants, occasionally additional EG-positive cells are labelled at ectopic positions close to the midline (white arrowhead). Their reproducible position suggests that they derive from a row 2 or row 3 NB. (E) In *DER* mutant embryos, the EG/EN-positive NB 7-3 cluster is missing in most hemineuromeres (asterisks). (F,G) The early anti-REPO staining pattern in *DER* mutants (G) is indistinguishable from wild type (F) indicating that the fate of the lateral glial precursor (arrows) is not affected in the mutant.

following, our conclusions from the transplantation experiments are mainly based on the comparison among these subclasses, rather than individual types of lineages.

From DER mutant cells, we obtained 59 identifiable CNS clones. Among these were 24 midline clones and 35 clones derived from NBs (I in Table 1) The composition of the identified lineages appeared normal suggesting that, in these cases, DER was dispensable for the formation and specification of the corresponding NBs as well as differentiation of their progeny. However, the data also uncovered a number of abnormalities. The ratios among the clones corresponding to the medial, intermediate and lateral group of NB lineages differed between mutant and control cells. Whereas the proportion of mutant cells that assumed the fate of medial NBs (21 out of 35 NB clones) corresponded to wild type (60% of identifiable NB clones), they gave rise to only few clones (n=5) derived from an intermediate NB (14%, compared to 40% in wild type). Thus, in the absence of DER function, the ability of vNE cells to assume the fate of intermediate NBs appears to be reduced. Furthermore, whereas wild-type cells isotopically transplanted into the vNE do not produce lateral NB lineages (see also Bossing et al., 1996), we obtained several lateral NB lineages from mutant cells (26%; see Table 1). This shows that a fraction of the mutant vNE cells becomes incorrectly specified to assume the fate of lateral NBs.

DER is dispensable for the formation of lateral NB lineages

Having shown that DER affects vNE cell fates, we next tested whether DER might also play a role in the specification of NBs derived from the dorsal part of the NE (dNE). The current model of DER activation is that signalling occurs in a graded fashion with high DER activity in ventral and low DER activity in more lateral positions of the neuroectoderm (Golembo et al., 1996a; Schweitzer and Shilo, 1997). In a second set of transplantation experiments, cells were taken from the dNE of flb^{IK35} donors and isotopically transplanted into wild-type hosts (30-50%VD; II in Fig. 1). This region of the NE normally gives rise to the lateral NB lineages (Schmidt et al., 1997). From DER mutant cells, we obtained 7 identifiable CNS clones that all showed the characteristics of lateral NB lineages (II in Table 1). Thus, these lateral NB lineages can be formed in the absence of DER function.

Taken together the analysis of lineages derived form isotopically transplanted cells from different parts of the NE reveals that medial and lateral NB lineages can form, whereas the ability to form intermediate NB lineages is disturbed when DER is cell autonomously absent. Furthermore, a proportion of cells from the vNE tends to assume the fate of lateral NBs. These findings are corroborated by the patterns of expression of cell-specific markers (see below).

Effects of DER on the expression of cell-specific markers

As a further approach to test our findings on DER function in dorsoventral CNS patterning, we stained embryos of the flb^{IK35} strain with cell-specific molecular markers. Mutant embryos (at stage 10/11) were stained with antibodies labeling specific sets of neurons or glial cells that have been previously assigned to individual neuroblasts (NBs) and their dorsoventral origin in the NE.

Among the cells in the wild-type ventral nerve cord that are labelled by an antibody against EVEN-SKIPPED (EVE) are progeny of two medial NBs, the aCC/pCC neurones (NB 1-1) and the CQ and fpCC neurones (NB 7-1; Fig. 2A; Broadus et al.,

1995; Bossing et al., 1996). In addition, the first ganglion mother cell (GMC4-2a) of the intermediate NB 4-2 and the resulting RP2/RP2-sib neurones (Doe, 1992) are stained. In *DER* mutants, the aCC, pCC, CQ and fpCC neurons are generally formed (Fig. 2B). In 10% of the examined hemineuromers (*n*=134), we found abnormalities in that EVE-positive cells in the NB 1-1 or NB 7-1 position were missing. Strikingly, however, we found that 97% of the examined hemineuromers (*n*=240) lacked the EVE-positive cells in the RP2/RP2-sib position. Also at earlier stages, we did not detect an EVE-positive cell at the position of GMC4-2a (Fig. 2B). Thus, in DER mutant embryos, EVE-expressing progeny of the intermediate neuroblast NB 4-2 were missing or

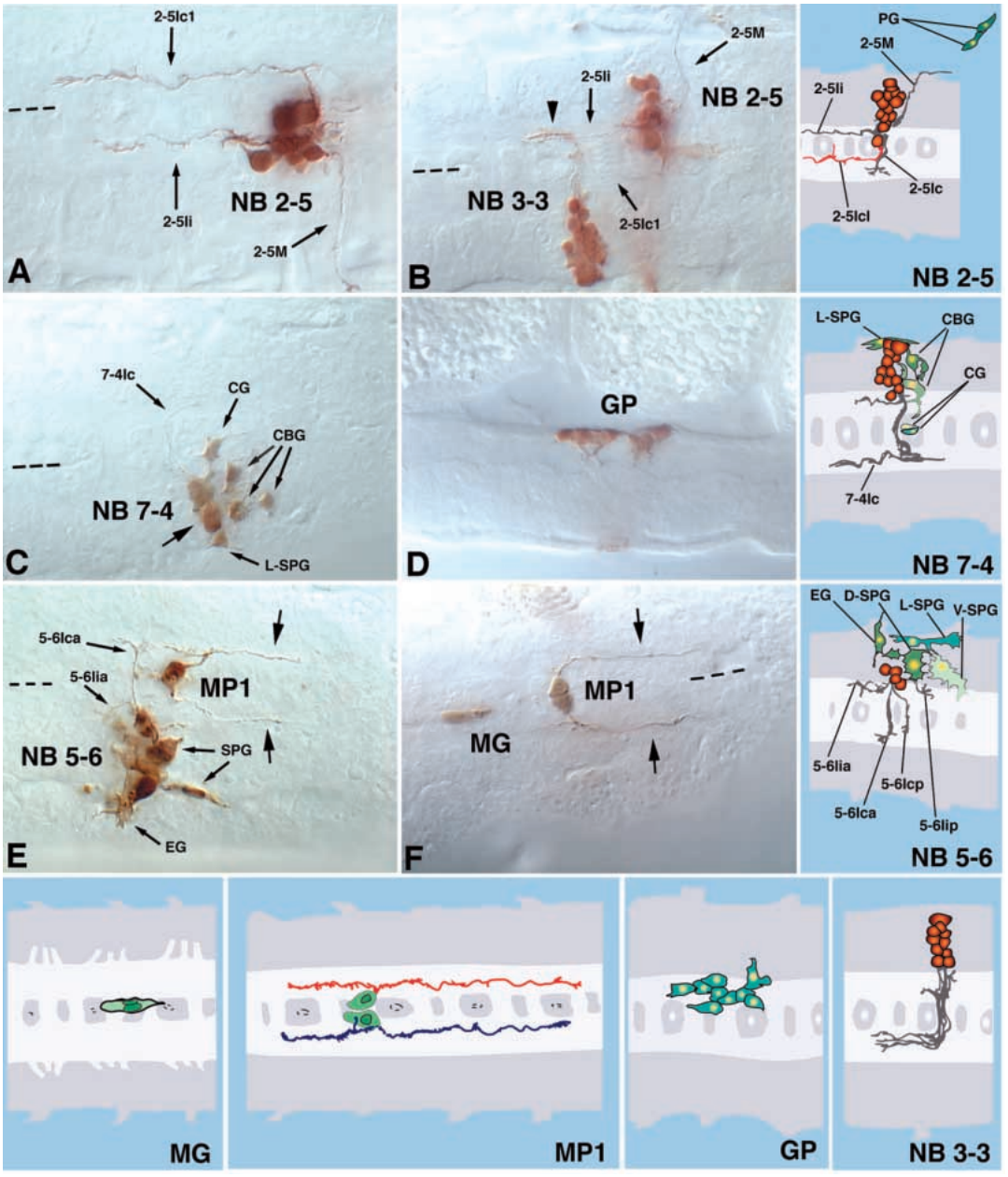

Fig. 3. DER is necessary for heterotopically transplanted dNE cells to assume ventral NB identities, but is not required to assume midline cell fate. (A-F) HRP-labelled clones; horizontal (A-C, E, F) and lateral (D) views of whole-mount preparations (stage 16/17); anterior to the left; orientation of the CNS midline is marked by stippled lines at the margins of A-C, E, F. Cells were removed from the dNE (30-50% VD) of *DER* mutant donors and were heterotopically transplanted into the vNE of wild-type hosts (III in Fig. 1; Table 1). Schematic drawings of wild-type lineages (see Bossing and Technau, 1994; Schmidt et al.; 1997) are shown for comparison. (A-E) *DER* mutant precursors developed according to their position of origin in that they produced lineages typical for NBs from the dNE, as opposed to wild-type cells, which under the same conditions assume ventral NB fates. (A) NB 2-5 clone, consisting of about 15 neurons with ipsilateral and contralateral interneuronal (2-5Ii and 2-5Ic1) and an ipsilateral motoneuronal projection (2-5M) and 2 peripheral glia cells (out of focus). (B) Two cells have been implanted that developed into NB 3-3 and NB 2-5 lineages. The visible projections 2-5Ii, 2-5Ic1 and 2-5M of the NB 2-5 clone are indicated (compare A). The NB 3-3 clone consists of about 12 neurons with a contralaterally projecting interneuronal fascicle (arrowhead) and an epidermal subclone (out of focus) (C) NB 7-4 clone, consisting of a cluster of about 8 neurons (big arrow) and 5 glial cells (CG, channel glia; CBG, cell body glia; L-SPG, lateral subperineurial glia). The contralateral interneuronal fascicle 7-4Ic is indicated. (D) GP clone, consisting of about 8 longitudinal glial cells on top of the connective. (E) Two cells have been implanted close to the midline. One of the cells developed as the lateral NB 5-6 lineage, which consists of about 5 neurons with ipsilateral and contralateral interneuronal projections (5-6 Iia and 5-6Ica are indicated) and about 3 glial cells (EG, exit glia; SPG, subperineurial glia). The second cell became part of the ventral midline and produced a midline lineage, MP1, consisting of two interneurons with ipsilateral projections (big arrows). (F) Two cells have been implanted into the midline that both assumed midline cell fate: one of them gave rise to a clone of two midline glia cells (MG) and the other to an MP1 clone (compare E). The posterior branches of the MP1 projections are fully differentiated (arrows), whereas the anterior branches are reduced compared to wild type or are not completely stained.

their fates were altered, while those of the two medial neuroblasts NB 1-1 and NB 7-1 seem to be only weakly affected.

As a further cell-specific marker, we used an antibody against the EAGLE (EG) protein (Dittrich et al., 1997). In wild-type embryos, EG is expressed in one intermediate (NB 7-3) and three lateral NBs (NB 2-4, NB 3-3, NB 6-4) and all their progeny (Higashijima et al., 1996; Dittrich et al., 1997; Fig. 2C). In DER mutant embryos (late stage 11), we could not detect EG-positive NB 7-3 progeny in 56% of the hemineuromers (n=174; Fig. 2D,E). In a few cases (3%, n=94), we found ectopic EG expression close to the ventral midline, a position where in wild-type embryos EG staining never appears (Fig. 2D). Double stainings for EG and ENGRAILED (EN) in flb^{IK35} mutant embryos reveals that the ectopic EG cells only express the EG but not the EN protein (Fig. 2D). With regard to its anterior/posterior position, the ectopic EG cell is always located at the level of row 2 or row 3 NBs. Taken together, these results suggest that, in DER mutant embryos, the intermediate NB7-3 is lacking in most cases, whereas a medially located NB lineage can be sometimes incorrectly specified in that it expresses a genetic marker typical of a more lateral NB cell fate. On the contrary, we did not detect any obvious changes in the staining pattern of the lateral NB 2-4, NB 3-3 and NB 6-4 lineages.

At stage 11, anti-REVERSED POLARITY antibody (REPO; Xiong et al., Campbell et al., 1994; Halter et al., 1995) is a specific marker for the lateral glioblast (GP; Fig. 2F) which gives rise to the longitudinal glia cells (Jacobs et al., 1989; Halter et al., 1995), and an antibody against the LADYBIRD EARLY protein (LBDE; Jagla et al., 1997) labels the lateral NB 5-6 and its progeny (J. U., unpublished data). In DER mutants (stage 11), we did not find any effects on the expression of these markers in the GP (Fig. 2G) or NB 5-6 lineages (data not shown).

The antibody stainings are in line with the results obtained from the isotopic transplantation experiments in that they show that loss of DER function mainly affects progeny of intermediate NBs and to a lesser extent progeny of medial NBs, whereas lateral lineages are unaffected.

DER is necessary in transplanted dorsal NE cells to respond to ventralizing signal(s)

In a further set of transplantation experiments, we tested whether DER is involved in non-autonomous ventralizing signalling. As previously shown by heterotopic transplantation experiments in wild-type embryos, signals exist in the NE that assign NBs to ventral identities. When cells are transplanted from dorsal sites of the NE (dNE; 30-50% VD) to ventral sites (vNE; 0-20% VD), the cells develop according to their new position in that they take over the fate of medial and intermediate NBs or CNS midline precursors (Udolph et al., 1995). Since DER appears to influence CNS patterning by primarily affecting the fate of vNE cells as outlined above, we tested whether this receptor might be necessary for the perception of the ventralizing signal(s). We heterotopically transplanted cells from the dNE of flb^{IK35} donors (gastrula stage) into vNE and midline of wild-type hosts (same stage; III in Fig. 1). We obtained 34 identifiable CNS clones from DER mutant cells. 23 clones could be assigned to specific NBs (III in Table 1). Strikingly, almost all of these 23 clones corresponded to lineages of lateral NBs (96%, Table 1; Fig. 3A-E). This is in clear contrast to the behavior of transplanted wild-type cells, which, under the same experimental conditions, exclusively produce medial and intermediate NB lineages (in addition to midline lineages; III in Table 1; see also Udolph et al., 1995). Thus, DER is necessary for the induction of heterotopically transplanted dNE cells to take over the identity of medial and intermediate NBs.

Induction of CNS midline cell fate does not require DER

Among the identified lineages obtained in the heterotopic transplantation experiment were another 11 clones that corresponded to lineages of CNS midline precursors. Thus, in contrast to the inability of DER-deficient dNE cells to take over the identities of medial and intermediate NBs, they can still produce midline lineages with about the same efficiency as transplanted wild-type cells (III in Table 1; Fig. 3E,F; see also Udolph et al., 1995). This demonstrates that the induction of midline cell fate per se does not require DER signalling. Consequently, additional signalling mechanism(s) must exist, which can induce midline cell fate independent from DER in the early gastrula embryo.

DISCUSSION

In previous work, we provided evidence for (a) ventralizing signal(s) within the neuroectoderm (NE) that is able to firmly commit a cell autonomous ventral identity during the early stage of gastrulation (Udolph et al., 1995). Here we now show that DER, which belongs to the group of receptor tyrosine kinases (RTK), is involved in receiving a part of the postulated ventralizing signals and is capable of cell autonomously specifying medial and intermediate NB cell fates. Our data suggest that the early NE exhibits a dorsoventral prepattern largely independent of the DER pathway, but that its further refinement via DER function is crucial to allow the formation and/or specification of intermediate NBs. Although, under experimental conditions, DER activity is also able to mediate medial NB identity cell autonomously, it seems to be less important for the development of medial NB lineages under normal conditions. On the contrary, DER appears to be dispensable for the formation of lateral NB lineages. Finally, we provide evidence for DER-independent signalling in the midline region, which is able to confer CNS midline identity.

Similar conclusions were reached by J. Skeath (1998) who analyzed the pattern of NBs and neuroectodermal domains in DER mutants using molecular markers.

Dorsoventral subpopulations of NBs depend on DER to different degrees

The results of our isotopic cell transplantation experiments in conjunction with the antibody stainings show that DER activity is most crucial for the development of intermediate NBs. Intermediate NB lineages like NB 4-2 and NB 7-3 are severely affected by the loss of DER function. A role of DER in specifying intermediate NB cell fates is also reflected by the expression pattern of the *escargot* (*esg*) gene (Yagi and Hayashi, 1997). *esg*, which encodes a zinc finger transcription factor (Whiteley et al., 1992), is expressed in two longitudinal stripes on each side of the wild-type embryo covering the medial and lateral regions of the NE. It is repressed specifically in the intermediate column of the NE. In DER mutants, however, *esg* becomes derepressed and can be found all over

the dorsoventral axis of the NE already at stage 6 (Yagi and Hayashi, 1997). Furthermore, it is interesting to note that, in tissue culture cells, *esg* interferes with transcriptional activation function of the proneural SCUTE protein (Fuse et al., 1994). Another possible target regulated by the DER pathway is the homeobox gene *msh* (*muscle segment homeobox;* Lord et al., 1995; D'Alessio and Frasch, 1996; Isshiki et al., 1997) which is allelic to *lottchen* (Büscher and Chia, 1997; Büscher and Chia, personal communication). Activation of the DER pathway is necessary to restrict MSH to the dorsal region of the NE (D'Alessio and Frasch, 1996), thereby prohibiting the expression within the more ventral regions of the NE. Ectopic expression of *msh* in the ventral NE affects the lineages of NBs derived from that region severely (Isshiki et al., 1997), so that the DER-mediated repression of *msh* is crucial to allow normal ventral NE fate development.

The medial NBs seem to be less DER dependent compared to the intermediate NBs. The antibody stainings in *DER* mutants show an exceptional loss of EVE-positive medial NB progeny. Furthermore, the isotopic transplantations show that a fraction of the DER mutant cells derived from the ventral NE becomes misspecified to follow the fate of lateral NBs. This is further supported by the ectopic medial expression of EG, which we found in rare cases in DER mutant embroys. However, the heterotopic transplantation experiments clearly show that DER activity is sufficient to induce medial NB fates. Therefore we conclude that DER activity is involved in medial NB fate determination, although to a lesser extend. It seems very likely that this is due to the redundant function of (a) DER-independent ventral patterning gene(s).

In contrast to medial and intermediate NBs, lateral NBs show no obvious phenotype in *DER* mutants. However, upon heterotopic transplantation of wild-type cells from the dorsal ectoderm (outside the NE; 80-100% VD) into wild-type dNE (30-50% VD), they developed a lateral NB fate according to their new position (data not shown). This finding together with the ubiquitous expression of DER in the blastodermal embryo reported by Zak et al. (1990) would be compatible with the possibility that graded activation of DER in the NE by its ligands might also contribute to the development of lateral NB fates. However, cells taken from the dorsal ectoderm (80-100% VD) of *DER* mutant donors and implanted into the dNE of wild-type hosts also developed region-specific lateral NB cell lineages (data not shown). This rules out that DER signalling is required in specifying lateral NB fates. Instead, this experiment points to the existence of other yet unknown signals controling lateral NB fates. However, we cannot rule out the possibility that NBs outside the domain of ventral induction follow a lateral default fate.

Aspects of dorsoventral CNS patterning independent from DER signalling

Although loss of DER function has a severe effect on intermediate NBs, the NE still shows a D/V polarity since the lateral and most of the medial NB lineages appear to be normally determined. This DER-independent identity is clearly a cell autonomous property: cells from the vNE transplanted into dNE retain their ventral identity (Udolph et al., 1995) and *DER* mutant cells from the dNE still develop into a lateral fate when transplanted ventrally into wild-type hosts (this work). Thus, this patterning is not only largely DER independent but is also already laid down at the time of transplantation (stage 7), i.e. before NB segregation. In accordance with this, there is a growing list of genes that are expressed in certain dorsoventral domains within the NE even before this stage. All of them are directly and/or indirectly dependent on the gradient of nuclear DORSAL protein. For example, *ventral nervous system defect* (*vnd*/NK2) (Mellerick and Nirenberg, 1995; Jimenez et al., 1995) encoding a homeobox protein is expressed specifically in the ventral part of the NE before gastrula stage. Its early expression is DER independent (Gabay et al., 1996) and relies on the early dorsoventral patterning genes such as *dorsal* (*dl*), *snail* (*sna*), *twist* (*twi*) and *decapentaplegic* (*dpp*) (Mellerick and Nirenberg, 1995). Loss of *vnd* leads to loss of ventral proneural clusters and consequently to a loss of medial NBs (Jimenez and Campos-Ortega, 1990; Skeath et al., 1994; Jimenez et al., 1995). Thus, *vnd* is a good candidate for a DER-independent ventral patterning gene.

Strinkingly, despite the requirement of DER for intermediate and medial NB development, the determination of the adjacent mesectoderm to form the CNS midline is independent from DER signalling. Whereas cells taken from the dNE of DER mutant donors are unable to take over the fate of medial NBs upon heterotopic transplantation into the vNE, they are induced to assume CNS midline cell fates upon heterotopic transplantation into the ventral midline. Thus, this capacity requires signalling mechanisms that are independent from DER. The nature of these mechanisms remains to be elucidated. There are indications that expression of *single minded*, the master gene of CNS midline development, is controlled in part by inductive influences mediated by the neurogenic gene *Notch* (Menne and Klämbt, 1994; Martin-Bermudo et al., 1995).

DER signalling is able to play an instructive role with respect to dorsoventral NB specification.

The obvious existence of DER-independent dorsoventral patterning of the NE raises the possibility that DER activity has a permissive rather than an instructive function, allowing NB development to occur according to independently laid down dorsoventral positional information. Although we cannot rule out that this is the case, our heterotopic cell transplantation experiments clearly show that DER signalling has the capacity to play an instructive role in this context: cells transplanted from the dNE into vNE switch their fate only due to the cell autonomous activity of the DER pathway (Udolph et al., 1995 and this work). We do not know yet, how this is achieved. One possibility is that DER is able to modulate patterning genes like *msh* and *esg* according to the new position of the cell. Alternatively, the DER pathway might be able to bypass these patterning mechanisms by regulating the new fate more directly.

Different phases of DER activation during NE patterning

Cells of the dNE that are heterotopically transplanted into the vNE adjust their development according to their new position due to the cell autonomous activation of the DER pathway. Thus, the transplanted cell must be able to interpret its new position based on a higher concentration of (an) activating DER ligand(s) compared to its original position.

Our data suggest that proper development of the vNE requires at least two phases of DER signalling: an early phase,

which is independent of the midline, and a later midline-dependent phase.

Previous data have concentrated on the midline-dependent phase of DER signalling. Two ligands are known that activate DER during embryogenesis: VEIN, a neuregulin like molecule (Schnepp et al., 1996) and SPITZ (SPI), a TGF-α homologue. SPI, which is processed by the transmembrane proteins RHOMBOID and STAR in the midline cells to produce the active secreted form (sSPI), has been shown to control dorsoventral patterning of the ventral epidermis by gradual activation of DER at stage 9/10 (Bier et al., 1990; Kolodkin et al., 1994; Schweitzer et al., 1995a,b; Golembo et al., 1996b). At this stage, the midline appears to be the only source of active DER ligand for the ventral ectoderm. Although there are indications that sSPI can diffuse up to five cell rows, DER activation has been observed in only three to four neuroectodermal cell rows on each side, with the highest level of activity in cells adjacent to the midline (Gabay et al., 1997a,b). It has been also previously shown that differentiation and/or maintenance of a subpopulation of cells in the CNS cortex requires a normal midline (Sonnenfeld and Jacobs, 1994; Menne et al., 1997). Furthermore, isotopic transplantation (stage 7) of midline cells that overexpress SPI leads to the formation of additional aCC/pCC neurons (Menne et al., 1997).

On the contrary, a number of observations point to the existence of early, midline-independent signals. First, the population of intermediate NBs which is preferentially affected in DER mutants at least partially derives from regions of the NE outside the range of SPI diffusion (see above). The fact that heterotopically transplanted cells can adjust to an intermediate NB fate due to DER function suggests, that DER can be activated in the intermediate NE region and has a direct role in this context. Second, in *single minded* mutants that lack midline-derived sSPI, no loss of RP2 neurons that derive from the intermediate NB 4-2 can be detected. Third, whereas local overexpression of sSPI in the midline (from stage 7) leads to additional aCC/pCC neurons, no effect has been observed on other identified neurons, like the RP2 neurons (Menne et al., 1997). This is in marked contrast to our finding that *DER* mutants show a nearly complete loss of RP2.

Gabay et al. (1997a,b) have recently shown that, before gastrulation (stage 5/6) DER is indeed broadly activated within a region of the vNE that corresponds to the early RHOMBOID expression domain and which probably includes the region from which the intermediate NBs originate. During gastrulation, this activity pattern is more and more restricted towards the midline. Furthermore, *vein* is expressed in blastoderm embryos in two ventrolateral stripes that are brought to the midline as gastrulation proceeds and genetic data suggest it acts together with *spi* to achieve the required level of DER activation for normal development of ventrolateral cells (Schnepp et al., 1996). Thus, for the early neuroectodermal DER function the midline is probably not the source of active ligands. The short-time expression of RHOMBOID and VEIN in more lateral positions and subsequent restriction to ventral sites could lead to a gradient of activating ligands that might be still present at stage 7, when cell transplantations were performed.

Principle mechanisms underlying ventral nerve cord patterning in insects show many parallels to the development of the neural tube in vertebrates. Homologous dorsoventral patterning genes control the specification of the neurogenic region of the ectoderm (Arendt and Nübler-Jung, 1996; Bier, 1997) and further spatial subdivision of the neuroectoderm (D'Alessio and Frasch, 1996). In both, *Drosophila* and vertebrates subsequent selection of neural precursor cells from the neural epithelium is mediated by two groups of genes, the proneural and the neurogenic genes (e.g. Campos-Ortega, 1993; Chitnis et al., 1995; Chitnis and Kintner, 1996; Dornseifer et al., 1997). The CNS midline cells in *Drosophila* like the vertebrate floor plate act as important organizing centers; in both systems the development of specific neural cells in the CNS cortex depends on the midline (Yamada et al., 1993; Hynes et al., 1995; Menne et al., 1997). Here we have shown that DER signalling controls the specification and differentiation of certain populations of cells in the *Drosophila* ventral nerve cord. This again resembles the situation in vertebrates: as shown recently, the timing and level of EGF receptor activation participates in the regulation of progenitor cell fate and proliferation during cortical development in mammals (Reynolds and Weiss, 1996; Burrows et al., 1997).

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