Segment polarity and DV patterning gene expression reveals segmental organization of the *Drosophila* brain

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Accepted 4 April 2003

**SUMMARY**

The insect brain is traditionally subdivided into the trito-, deuto- and protocerebrum. However, both the neuromeric status and the course of the borders between these regions are unclear. The *Drosophila* embryonic brain develops from the procephalic neurogenic region of the ectoderm, which gives rise to a bilaterally symmetrical array of about 100 neuronal precursor cells, called neuroblasts. Based on a detailed description of the spatiotemporal development of the entire population of embryonic brain neuroblasts, we carried out a comprehensive analysis of the expression of segment polarity genes (*engrailed*, *wingless*, *hedgehog*, *gooseberry distal*, *mirror*) and DV patterning genes (*muscle segment homeobox*, *intermediate neuroblast defective*, *ventral nervous system defective*) in the procephalic neuroectoderm and the neuroblast layer (until stage 11, when all neuroblasts are formed). The data provide new insight into the segmental organization of the procephalic neuroectoderm and evolving brain. The expression patterns allow the drawing of clear demarcations between trito-, deuto- and protocerebrum at the level of identified neuroblasts. Furthermore, we provide evidence indicating that the protocerebrum (most anterior part of the brain) is composed of two neuromeres that belong to the ocular and labral segment, respectively. These protocerebral neuromeres are much more derived compared with the trito- and deutocerebrum. The labral neuromere is confined to the posterior segmental compartment. Finally, similarities in the expression of DV patterning genes between the *Drosophila* and vertebrate brains are discussed.

Key words: CNS, Brain development, Neuroblasts, Segment polarity genes, Dorsoventral patterning genes, Segmentation, *Drosophila*

**INTRODUCTION**

In order to integrate multiple sensory input and generate appropriate behavioural responses, the central nervous system (CNS) has to be composed of region-specific structures that fulfill particular functions. The formation of these structures can be correlated back to the activity of patterning genes during early embryonic development. Molecular and genetic tools as well as manipulation techniques make *Drosophila* a suitable model system for the investigation of developmental processes that underlie patterning and cellular diversity in the CNS. So far, investigations on CNS development in the *Drosophila* embryo have mainly focused on its less complex truncal region – the ventral nerve cord. The ventral nerve cord arises from multipotent stem cells, called neuroblasts (NBs), which delaminate from the ventral neurogenic region in a segmentally repeated pattern (Doe, 1992; Hartenstein and Campos-Ortega, 1984). Segmental patterning defines functional units, which can then be refined during further development. For example, within a segment (neuromere) each NB acquires a unique identity and produces a specific cell lineage (for a review, see Doe and Technau, 1993). Genetic mechanisms of neuromere and NB formation in the ventral nerve cord are quite well understood (for a review, see Campos-Ortega, 1993), and some advances in understanding the processes that lead to the specification of individual NB identities have recently been made. Specific identities appear to be conferred to presumptive NBs in the neuroectoderm by positional cues. For example, the segment polarity genes subdivide the neuroectoderm into transverse rows along the anteroposterior axis and the dorsoventral patterning genes into longitudinal columns along the DV axis. The superimposition of the expression patterns of both gene groups establishes a Cartesian coordinate system of positional cues in which the fate of a particular NB depends on the respective ‘quadrant’ in which it is formed (for reviews, see Bhat, 1999; Skeath, 1999).

The situation is much more complex in the procephalic neuroectoderm and the brain. The insect brain develops highly organized neuropil structures, such as the mushroom bodies and the central complex (e.g. Bullock and Horridge, 1965; Hanesch et al., 1989; Hanström, 1928; Strausfeld, 1976), that are required for behavioural functions such as olfactory learning and memory or the control of locomotor activity (e.g. Heisenberg, 1998; Strauss and Heisenberg, 1993); these structures have no equivalents in other ganglia. The key towards elucidating the origin of these structures lies in an understanding of the segmental organization of the brain. However, the segmental pattern in the head is highly derived
and its metameric organization has been intensely debated (e.g. Boyan and Williams, 2000; Haas et al., 2001; Hirth et al., 1995; Jürgens et al., 1986; Rempel, 1975; Rogers and Kaufman, 1996; Schmidt-Ott et al., 1994). In Drosophila the expression of engrailed and wingless argues for the existence of four pregnatal segments: the intercalary, antennal, ocular and labral segments (Schmidt-Ott et al., 1994; Schmidt-Ott et al., 1995; Schmidt-Ott and Technau, 1992). Although it has been suggested that each head segment contributes to the brain (Schmidt-Ott and Technau, 1992), the arrangement and boundaries of the corresponding neurones, and the origin and identities of their progenitor cells are largely unknown.

Based on a detailed description of the entire population of brain NBs and their spatiotemporal pattern of segregation from the neuroectoderm (Urbach et al., 2003), we have investigated the expression of segment polarity genes and dorsoventrally patterning genes in the procephalic neuroectoderm, as well as in the individually identified brain NBs through to stage 11, when the full complement of NBs has formed. The work provides new insight into the positional cues expressed in the procephalic neuroectoderm and the segmental organization of the evolving brain. The data strongly support the view that the pregnatal Drosophila head is composed of four segments, and we now attribute to each of the four pregnatal segments a corresponding neurone. Furthermore, we provide evidence that the protocerebrum consists of two neurones, which derive from the ocular and labral segment. The segmental character of these neurones is less conserved compared with the trito- and deutocerebrum, deriving from the intercalary and antennal segment. Finally, we discuss similarities in the expression of dorsoventrally patterning genes between the Drosophila and vertebrate brain.

MATERIALS AND METHODS

Drosophila strains

The following fly strains were used: Oregon R (wild type), engrailed-lacZ (ryXho25) (Hama et al., 1990), hedgehog-lacZ (16E) (Mohler et al., 1995) (kindly provided by J. Mohler), mirror-lacZ (Broadus et al., 1995; McNeil et al., 1997) (kindly provided by H. McNeil and M. Simon), muscle segment homeobox-lacZ (thh96) (Ishiki et al., 1997) (kindly provided by A. Nose), ventral nervous system defective-lacZ (kindly provided by F. Jimenez) and wingless-lacZ (Broadus et al., 1995).

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); additionally, we used the trunk NB pattern (Doe, 1992) as a further morphological marker for staging. 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-Asense (1:5000) (Brand et al., 1993) (kindly provided by Y.-N. Yan), rabbit-anti-Deadpan (1:300) (Bier et al., 1992) (kindly provided by H. Vaessen), 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-Injected (4D9:1:4) (Patel et al., 1989) (Developmental Studies Hybridoma Bank), 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-Ventral nervous system defective (1:2000) (McDonald et al., 1998) (kindly provided by F. Jimenez) and mouse-anti-Wingless (1:10, Developmental Studies Hybridoma Bank), anti-DIG-AP (1:1000, Roche). The secondary antibodies (Dianova) were either biotinylated (goat anti-mouse, goat anti-rabbit) or alkaline phosphatase-conjugated (goat anti-mouse, goat anti-rabbit, goat anti-rat) and diluted 1:500.

Whole-mount in situ hybridization

DIG-labelled intermediate neuroblast defective (ind) RNA probe (kindly provided by M. P. Scott) was synthesized with T7 polymerase and HindIII linearized pNB40-ind as a template 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 6.0. Semi-schematic presentations are based on camera lucida drawings.

RESULTS

The metameric expression of segment polarity genes is conserved in the early brain

In the trunk neuroectoderm, segment-polarity genes are expressed in stereotypical segmental stripes, and in NBs that delaminate from these domains, subdividing each neurone along the AP axis (Bhat, 1996; Broadus et al., 1995; Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Zhang et al., 1994). In the pregnatal head region the expression domains of segment polarity genes are less obvious, but previous analysis of engrailed and wingless expression in the head peripheral ectoderm, and of PNS mutant phenotypes, support the existence of four pregnatal segments in Drosophila: the intercalary, antennal, ocular and labral segments (Schmidt-Ott et al., 1994; Schmidt-Ott et al., 1995; Schmidt-Ott and Technau, 1992). However, the identity and organization of brain structures deriving from these segments is still obscure. In order to obtain evidence concerning the number and extent of the brain neurones, and to map the position of their boundaries, we analysed the expression of segment polarity genes, including wingless, hedgehog, gooseberry-distal, engrailed, injected and mirror. The spatiotemporal pattern of their expression was traced in the neuroectoderm and in the NB-layer until stage 11, when all brain NBs are formed. The data (detailed in Figs 1, 2 and 4) show that segmental expression is retained for most of the investigated segment polarity genes in both the developing head ectoderm (procephalon) and brain NBs, providing landmarks for the definition of segmental domains within the developing brain NB pattern.

engrailed (en) expression domains in the trunk define the posterior segmental compartments (DiNardo et al., 1985; Poole et al., 1985), from which NBs of row 6 and 7 and NB1-2 derive (Broadus et al., 1995). In the pregnatal head (neuroectoderm: Fig. 1B,C,E,F; Fig. 2A,C,E,I,K,L) (NBs: Fig. 1A,D; Fig.
2B,D,F,I,M) we find en expression as follows: from late stage 8 in the posterior ectoderm of the antennal segment (en/inv; red), hedgehog-lacZ (hh; purple), mirror-lacZ (mirr; blue), wingless-lacZ/Wingless protein (wg; green), and Gooseberry-distal protein (gsb-d; pink) at the level of identified brain NBs (A,D) and procephalic neuroectoderm (B,C,E,F). All panels show semi-schematic representations of a ventral view of the left half of head flat preparations; anterior (a) is towards the top and dorsal (d) is towards the left. Nomenclature of brain NBs is according to their position in the trito- (T), deutero- (D) and protocerebrum (P) (for details of the nomenclature see Urbach et al. (Urbach et al., 2003)). In (B,C,E,F), thick hatching marks strong expression and thin hatching marks weak expression of the respective gene; for comparison, NBs underlying the peripheral ectoderm are encircled by broken lines. The expression of en, gsb-d and wg is confined to intermediate and dorsal brain NBs (A,D) and corresponding sites of the peripheral procephalic ectoderm (B,C,E,F). By stage 10, Gsb-d is detected in the ectoderm of all four pregnathal head segments (E). In the gsb-d ocular domain (gsb-d oc) and two corresponding NBs (Ppd3, Ppd10) gsb-d expression is transient (compare with Fig. 2). It is also transiently expressed (between stages 10 and 11) in the clypeolabral ectoderm [in the gsb-d labral spot, which is partly colocalized with the wg labral spot (E)] from which no NBs emerge (see also Fig. 2). mirror-lacZ is not segmentally expressed in the procephalic neuroectoderm; it is observed in the invaginating foregut and flanking neuroectoderm, and is additionally detected in a more intermediate ectodermal antennal spot (from which Dd6 arises). A large wg domain extends from the antennal into the ocular head region (B), but later separates into an antennal stripe (wg as) and ocular head blob (wg hb) (E). hh-lacZ expression accumulates in posterior regions of the antennal and ocular ectoderm (F; see also Fig. 2J). (A,D) Note that a large number of identified brain NBs (especially protocerebral) do not express any segment polarity gene. For a detailed description, see text. Stars indicate mandibular NBs. as, en antennal stripe; CL, clypeolabrum; FG, foregut; gsb-d as, gsb-d antennal stripe; gsb-d is, gsb-d intercalary stripe; gsb-d lr, gsb-d labral spot; gsb-d oc, gsb-d ocular domain; hs, en head spot; is, en intercalary stripe; ML, ventral midline; wg as, wg antennal stripe; wg hb, wg ocular head blob [for nomenclature of en and wg expression domains in the procephalic ectoderm, see Schmidt-Ott and Technau (Schmidt-Ott and Technau, 1992)].
Fig. 2K). By contrast, the En-positive antennal stripe and head spot are only subfractions of the large hh-lacZ domain, which, between stage 9 and 10, encompasses the antennal segment and the posterior part of the ocular segment. We find that all NBs delaminating from this domain express hh-lacZ (Fig. 1A,D). From stage 10 onwards, en expressing NBs maintain a strong hh-lacZ signal, whereas hh-lacZ subsequently diminishes in the neuroectoderm and in NBs between the en antennal stripe and head spot (compare Fig. 1D,F with Fig. 2IJ). Additionally, hh-lacZ-expressing NBs positioned dorsally to the en/hh-lacZ-coexpressing Ppd5 and Ppd8 (both NBs demarcating part of the posterior border of the ocular neuromere), appear to prolong the boundary between the deutono- and protocerebrum in the dorsal direction (Fig. 1D, Fig. 2I, Fig. 4).

From late stage 8 onwards, Wingless (Wg) protein is expressed in a neuroectodermal domain spanning a broad area
of the ocular and the anterior antennal segment (and in the invaginating foregut) (see also Baker, 1988; van den Heuvel et al., 1989). This becomes clearer in En/Wg double labelling at stage 9, revealing that the en hs is localized within this Wg domain (Fig. 1B). In contrast to earlier observations (Richter et al., 1998; Younossi-Hartenstein et al., 1996), we find that, at that stage, Wg is already detectable in about 4-5 protocerebral NBs (Pcd6, Pcd15, Pcd7, Ppd3; Fig. 1A), derived from the region with strongest Wg expression [which later corresponds to the wg head blob; for nomenclature of wg expression domains in the procephalic ectoderm, see Schmidt-Ott and Technau (Schmidt-Ott and Technau, 1992)]. Furthermore, Wg is faintly expressed in the deutocerebral Dd7 (Fig. 1A) emerging from the antennal part of the Wg domain (Fig. 1B), which corresponds to the later wg antennal stripe (Fig. 1E, Fig. 2G,J). By stage 10, when the wg head blob is clearly distinguishable from the wg antennal stripe (Fig. 1E), about 10-12 Wg-positive NBs have emerged from this domain (Fig. 1D). In addition, we found a small, spot-like Wg domain in the intercalary segment (Fig. 1E; wg intercalary spot) from which a single NB (Td4) delaminates (Fig. 1D). Thus, all three Wg domains, the intercalary, antennal and ocular (head blob), contribute to the anlage of the brain. From late stage 9 an additional Wg domain is visible in the ectodermal anlage of the clypeolabrum (Fig. 1B,E, Fig. 2G,J), which is the Wg counterpart to the En/Inv-positive region in the ‘dorsal hemispheres’ [wg labral spot in Schmidt-Ott and Technau (Schmidt-Ott and Technau, 1992)]. Upon double labelling for either asense or deadpan (both are general markers for neural precursor cells) and Wg, in embryos between stage 9 and 11 we could not identify any NB emerging from the wg labral spot. By stage 11 the number of Wg expressing NBs originating from the ocular head blob has increased to about 16-20 (Fig. 2H,I), which is more than 25% of the total number of identifiable protocerebral NBs. Three Wg-positive NBs are identified in the deutocerebrum and one in the tritocerebrum (Fig. 2I).

The gooseberry (gsb) locus encodes two closely related proteins, Gsb-distal (Gsb-d) and Gsb-proximal (Baumgartner et al., 1987; Bopp et al., 1986), which are both expressed in the developing ventral nerve cord (Gutjahr et al., 1993; Ouellette et al., 1992). Gsb-d is segmentally expressed at high levels in all row 5 and 6 NBs, as well as in a median row 7 NB (NB 7-1) (Broadus et al., 1995; Zhang et al., 1994). We analysed the expression of gsb-d during early neurogenesis in the head region, and found segmental expression of Gsb-d to be conserved in parts of the prepharyngeal head ectoderm and deriving NBs (for details see Fig. 1A,B,D,E; Fig. 2C,D,I,J). Gsb-d/En double labelling show that the gsb-d intercalary and antennal stripes are expressed anteriorly to the corresponding en stripes, and are partly overlapping with the en stripes (Fig. 1B,E, Fig. 2C,J). Consequently, NBs from the posterior part of the gsb-d stripe in the tritocerebrum and deutocerebrum co-express en (Td3, Dd5; Fig. 1D, Fig. 2D,I), and those from the anterior part co-express Wg (Td4, Dd1 and Dd7; as seen in Gsb-d/Wg double labelling; Fig. 1A,D, Fig. 2I, and data not shown), resembling the situation in the ventral nerve cord. However, Dd8 and all Wg-positive protocerebral NBs do not co-express Gsb-d (except for Ppd3 which, like Ppd10, transiently expresses gsb-d during stage 10; Fig. 1D). Gsb-d can also be detected at a low level in ganglion mother cells of the respective NBs, but fades away in NBs and their progeny during germ band retraction. Expression of the protein in the brain is completely downregulated at stage 13 (data not shown).

In the trunk, mirror (mirr)-lacZ is expressed in segmental ectodermal stripes giving rise to mirr-lacZ-positive NBs of row 2 and several NBs that flank row 2 at stage 11 (Broadus et al., 1995; McNeill et al., 1997). The pattern of mirr-lacZ expression in the procephalic neuroectoderm and brain NBs differs significantly from the trunk. We find no evidence of a segmental arrangement of mirr-lacZ expression in the procephalon (for details, see Fig. 1A,C,D,F, Fig. 2E,F,I,K). Interestingly, regarding the DV axis, mirr-lacZ is mainly limited to the ventral part of the pNR and corresponding NBs (as confirmed by mirr-lacZ/Vnd double staining, although there is a faint dorsal mirr-lacZ expression, in the region of the later invaginating optic lobe anlage; Fig. 1A,C,D,F, Fig. 2K,L), and is, at stage 9/10, roughly complementary to en, Wg and gsb-d expression, the domains of which are mainly confined to...
intermediate and dorsal regions of the pNR (Fig. 1B,C,E,F). At stage 11, expression extends towards the dorsal part of the antennal neuroectoderm (Fig. 2E,K) and is observed in all NBs of the ventral deutocerebrum, as well as in two tritocerebral (Tv5, Td8) and four ventral, protocerebral NBs (Pad1, Pcv1, Pcv2, Pcv3; Fig. 2F,I). Although expression is also found in the clypeolabrum (Fig. 2E,K), we did not identify mirr-lacZ-positive labral NBs.

Expression of dorsoventral patterning genes during early brain development

In addition to the segment polarity genes, the dorsoventral patterning genes ventral nervous system defective (vnd), intermediate neuroblast defective (ind) and muscle segment homeobox (msh) have been shown to confer positional information to the truncal neuroectoderm, which also contributes to the specification of NBs (reviewed by Skeath,
Fig. 3. Expression of DV patterning genes at embryonic stages 9 (st9), 10 (st10) and 11 (st11). Colour code indicates the expression of msh (msh-lacZ and Msh protein), ind transcripts and vnd (vnd-lacZ and Vnd protein) in identified NBs (A,B,E) and the procephalic neuroectoderm (C,D,F). en expression domains are shown for comparison. Orientation of the semi-schematic representations is as in previous figures. (A,C) Stage 9; note the spot-like ind expression in the intercalary (ind is; ind intercalary spot), antennal (ind as; ind antennal spot) and ocular (ind oc; ind ocular spot) head region; the ind antennal spot, in contrast to the intercalary, overlaps completely with the ventral en domain; the emerging Dd1 does not express vnd. (B,D) Stage 10; vnd is already downregulated in part of the ventral antennal neuroectoderm and corresponding Dv3 and Dv6, and is also not expressed in the newly developed Dv5. msh and vnd expression overlaps in a small area of the antennal ectoderm and emerging Dd5. (E,F) Stage 11; note that most of the identified brain NBs (especially protocerebral NBs) do not express any DV patterning gene. (G,IK) Left half of head flat preparations double labelled for en expression (en-lacZ; Inv protein) and msh (msh-lacZ, Msh protein), ind (transcripts) or vnd (vnd-lacZ and Vnd protein), respectively; broken line marks the ventral midline; insets depict lateral views of stage 11 whole-mount heads. (HI,L) Close-ups of regions indicated in G,IK by frames (focus on the level of NBs). Immunopositive NBs are indicated by white, immunonegative NBs by black inscription. (G,H) msh-lacZ/Inv-antibody double labelling. The anterior border of the msh expression domain is positioned immediately posterior to the en hs (G), and runs between the deutocebral and ocular protocerebral NBs (H). (I,J) DIG ind mRNA/Inv antibody double labelling. (K,L) en-lacZ/Vnd antibody double labelling. Vnd protein has disappeared in most parts of the antennal neuroectoderm (K) and NBs (L); note the dorsally directed extension of vnd domains in the anterior and ocular procephalic regions. The antennal vnd expression overlaps dorsally with the En-positive Dv8 and Dd5. The posterior border of the ocular vnd domain runs between deuto- and protocerebral NBs (L). AN, IC, MD, MX, antennal, intercalary, mandibular and maxillary segment, respectively; CL, clypeolabrum; FG, foregut; ML, ventral midline; OA, Bolwig organ/optic lobe anlagen; as, en antennal stripe; cl, en expression in the clypeolabrum; hs, en head spot; is, en intercalary stripe; ind is, ind intercalary spot; ind as, ind antennal spot; ind oc, ind ocular spot.

1999). For the head and brain, a detailed analysis of the expression of these genes has not yet been undertaken. In order to elucidate their putative role in patterning the head and brain, we analysed the expression of vnd, ind and msh in the procephalic ectoderm and NBs in the early embryo (until stage 11). Although our data are consistent with their role in dorsoventral patterning being principally conserved in the procephalon, we also find significant differences in their patterns of expression compared with the trunk (as outlined in the following and in Figs 3, 4).

At the blastodermal stage, Ventral nervous system defective protein (Vnd) is expressed in bilateral longitudinal stripes corresponding to the most ventral neuroectodermal column (Jimenez et al., 1995; McDonald et al., 1998; Mellerick and Nirenberg, 1995), and is by stage 11 detected in all ventral and two intermediate NBs of the ventral nerve cord. Interestingly, the latter co-express en and are located in the posterior compartment of each truncal neuromere (Chu et al., 1998; McDonald et al., 1998; Shao et al., 2002; Uhler et al., 2002). At gastrulation the ventral longitudinal vnd domain reaches anteriorly across the cephalic furrow into the procephalic neuroectoderm (data not shown). By stage 9, vnd maps in the ventral neuroectoderm of the prospective intercalary, antennal and ocular segment (which is confirmed by En/Vnd double labelling; Fig. 3C) and is observed in ventral NBs of the antennal (Dv2, Dv3, Dv6) and ocular neuromere (Pcv1, Pcv3, Pcv6, Ppv2) (Fig. 3A). It appears as if the dorsal part of the Vnd-positive antennal neuroectoderm partly co-expresses ind at that stage (Fig. 3C), but the NB Dd1, which emerges from this ectodermal region expresses only ind and not vnd (Fig. 3A). This is possibly due to the transient expression of vnd in most parts of both the ventral antennal ectoderm (compare Fig. 3C with 3D) and corresponding NBs: by stage 10 Vnd is detected in the ventral Dv2, Dv4 and Dd5, but is already downregulated in Dv3 and Dv6, and by stage 11 it is confined to Dd5 and the new Dv8 (Fig. 3B,E,L). As a consequence of the downregulation of vnd (compare Fig. 3C,D,F,K), some ventral deutocerebral NBs, which delaminate between stage 9 and 11 from this domain were not observed to express vnd (e.g. Dv1, Dv5, Dv7; Fig. 3B,D,E,L). By stage 11 Vnd is seen in four tritocerebral (Tv2, Tv3, Tv4, Tv5), in two deutocerebral (Dd5, Dv8), and in a cluster of about 13 protocerebral NBs (Fig. 3E,L). Interestingly, vnd expression expands along the posterior border of the en intercalary stripe (en is), and is also significantly extended dorsally into the en antennal stripe (Fig. 3D,F,K) and the NBs delaminating from there. The fact that vnd and en are co-expressed in Tv5 and in Dd5, Dv8 (Fig. 3L, Fig. 4) is in agreement with findings in the ventral nerve cord, where these genes are co-expressed in two intermediate NBs (Chu et al., 1998; Shao et al., 2002). This indicates that vnd demarcates the ventral part of the posterior border in trunk as well as in brain neuromeres. Furthermore, the posterior border of the ocular vnd domain (including the NBs Pcv1, Pcv2, Pcv3, Ppv1, Ppv2, Ppv3) abuts dorsally the En-positive NBs Ppd5 and Ppd8 (deriving from the en head spot; Fig. 1D, Fig. 3E,K,L), supporting the view that these NBs demarcate the posterior border of the ocular neuromere (Fig. 4).

Intermediate neuroblast defective (ind) is expressed in the blastoderm in a bilateral longitudinal column (intermediate column neuroectoderm) just dorsal to the vnd domains. In the trunk, at stage 9 (when ind mRNA is no longer present in the neuroectoderm), it is expressed in all intermediate NBs and finally, at stage 11, it is confined to the NB 6-2 (Weiss et al., 1998). In the head, at stage 9, ind is detected in an intermediate longitudinal ectodermal domain in the intercalary segment (ind is; Fig. 3C), and weakly in an intermediate ectodermal patch in the antennal segment (ind as; Fig. 3C) as well as in the deutocerebral NB Dd1 which develops from this patch (Fig. 3A). At the same stage, we observed a further signal in a dorsal ectodermal patch of the ocular region (ind oc, Fig. 3C). The ectodermal ind patches in the intercalary, antennal and ocular segments are both separate from each other and from the ind domain in the trunk (Fig. 3C,D,I,F). Interestingly, ind mRNA is significantly longer present in the ectoderm of the intercalary and mandibular segment, when compared with the antennal segment and the trunk ectoderm (data not shown). This presumably mirrors the delayed onset of neurogenesis in both segments (see also Urbach et al., 2003). Until stage 10, five NBs derive from the three ind patches: Td1, Td2, Td3, from the intercalary, Dd1 from the antennal and Ppd13 from the ocular ind patch (Fig. 3B,D). Subsequently, the ocular ind patch enlarges but never reaches the ocular vnd domain (Fig. 3F), and by stage 11 about four additional Ind expressing NBs (Pcd7, Pcd13, Ppd6, Ppd9) are identifiable (Fig. 3D,E).
**Expression of DV patterning genes differs in the head and trunk neuroectoderm**

Comparing the expression of DV patterning genes in the trunk and procephalic region we observed the following significant differences.

1. Whereas *msh* is expressed in all segments of the trunk (Ishihiki et al., 1997), it is not expressed in the preantennal head ectoderm (Fig. 3C-H).

2. *ind* expression is a continuous stripe in the trunk, but forms three segmental patches in the procephalon. *ind* expression in the antennal segment appears to overlap with transient *vnd* expression [Fig. 3C; compare also with cell ‘cluster 1’ in McDonald et al. (McDonald et al., 1998)]. Yet, this ectodermal region gives rise to Dd1 which expresses *ind* but not *vnd* (Fig. 3A).

3. The *msh* and *vnd* domains partially share a common border in the intercalary and antennal segment by stage 9 (Fig. 3C), and furthermore show a partial overlap in the antennal ectoderm by stage 10/11 (Fig. 3D,H). The En-positive Dd5 co-expresses *msh* and *vnd* (Fig. 3B,E,H,L), whereas co-expression of *msh* and *vnd* was not observed in NBs of the ventral nerve cord (McDonald et al., 1998).

4. In the ocular segment the *ind* domain is separated from the *vnd* domain (Fig. 3C,D,F,I), whereas in the trunk neuroectoderm these domains are adjacent to each other.

5. *vnd* expression is dynamic and from stage 9 onwards is downregulated in parts of the antennal neuroectoderm and deutocerebral NBs (see *vnd* section).

6. More than half of the total number of identified brain NBs do not express any of these DV patterning genes. Most of these NBs derive from the preantennal segments (Fig. 3A,B,E).

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**Fig. 4.** Neuromeric model of the early embryonic brain. Based on the expression of the segment polarity genes *en/inv* and *hh* as well as the DV patterning genes *msh* and *vnd* we propose the pregnathal brain to consist of four neuromeres. Red lines indicate the borders between the tritocerebrum (Tc; comprising about 13 NBs), the deutocerebrum (Dc; comprising about 21 NBs), the ocular part of the protocerebrum (Oc-Pc; comprising about 60 NBs) and the labral part of the protocerebrum (Lr-Pc; comprising about 10 Inv-positive NBs). Note, the ventral part of the posterior border of the deutocerebrum is given by Dv2 and Dv4, which at stage 10 (st10) transiently express *vnd*. The neuromeric identity of Ppd2 is unclear; colour intensities indicate low (~) and high (+) expression levels of *en/inv* and *hh*. AN, IC, MD, MX, antennal, intercalary, mandibular and maxillary segment, respectively; CL, clypeolabrum; Dc, deutocerebrum; Lr-Pc, labral part of the protocerebrum; Oc-Pc, ocular part of the protocerebrum; Tc, tritocerebrum; FG, foregut; ML, ventral midline; OA, Bolwig organ/optic lobe anlagen.
This implies that other, still unknown factors might be involved in the DV patterning of the anterior head neuroectoderm and protocerebrum.

**Segmental boundaries in the early embryonic brain**

With regard to the expression of the segment polarity genes en, hh, wg and gsb-d, as well as the DV patterning genes msh and vnd, we propose that the procephalic (pregnathal) neuroectoderm gives rise to four brain neuromeres: the tritocerebrum, the deutocerebrum, the ocular and the labral neuromere. These tightly fused neuromeres form a supraoesophageal brain hemisphere on either side. The ocular and labral neuromeres represent the most prominent part of the brain which is traditionally referred to as the protocerebrum.

The detailed analysis of the dynamic expression of these genes in the procephalic neuroectoderm and in the identified brain NBs allows us to map the boundaries of the brain neuromeres (summarized in Fig. 4). The posterior border of the tritocerebrum is clearly represented by the en- and hh co-expressing NBs Tv4, Tv5, Td3, Td5. In the antennal and preantennal neuroectoderm the expression of en, hh, wg and gsb-d is largely restricted to intermediate and dorsal regions, and NBs deriving from there. Thus, regarding segment polarity genes, a clear demarcation of the antennal and preantennal neuromeres is only possible for the intermediate and dorsal, but not for the ventral domains. vnd is observed to be co-expressed with en in some tritocerebral (Tv5) and deutocerebral NBs (Dv8 and Dd5), located at intermediate DV positions. This is consistent with observations in the trunk, where vnd expression is dorsally expanded into each en domain in the neuroectoderm, as well as at the level of NBs (Chu et al., 1998). We therefore suggest that the (transiently) vnd expressing NBs Dv2 and Dv4, which follow Dd5 and Dv8 ventrally, demarcate the ventral part of the posterior border of the deutocerebrum. The intermediate part of this border is defined by the en/hh/vnd-co-expressing Dv8, Dd5, and the dorsal part by the en- and hh-co-expressing Dd9 and Dd13. For the posterior border of the ocular neuromere, we propose the following. Under the assumption that vnd expression also marks the posterior compartment in this neuromere, the vnd expressing NBs Pcv1, Pcv2, Pcv3, Pvp1, Pvp2 and Pvp3 would demarcate the ventral part of this border. The intermediate part is defined by the en/hh-co-expressing Pd5 and Pd8, and the dorsal part by the Hh-lacZ-positive NBs Ppd10, Ppd11, Ppd15 and Ppd16. Interestingly, the anterior border of the msh domain abuts exactly on the posterior ocular segmental border, indicating that msh expression is confined to the trito- and deutocerebrum. inv expression is observed in about 10 NBs deriving from the most anterior part of the protocerebral anlage, a region that corresponds to the En-positive ‘dorsal hemispheres’ (en dh) (Schmidt-Ott and Technau, 1992). We suggest that these NBs represent the neural correlate of the labral segment. The existence of a labral neuromere deriving from the en dh has already been discussed by Schmidt-Ott and Technau (Schmidt-Ott and Technau, 1992). This fourth brain neuromere seems to be of rudimentary character as it is confined to the posterior segmental compartment (considering that en/inv is normally expressed in the posterior compartment), and we did not find NBs anterior to en dh. Thus, the wg domain in the clypeolabral ectoderm, which is located immediately anterior to the en dh does not give rise to brain NBs (Fig. 2I,J). The existence of four brain neuromeres, in the spatial orientation shown, is furthermore substantiated by the segmental expression of other genes like gsb-d (Fig. 1D, Fig. 2J), sloppy paired 1 and ladybird (see Urbach and Technau, 2003).

**DISCUSSION**

**Reconstruction of neuromeric boundaries in the developing Drosophila brain**

In previous papers, based on the expression of the segment polarity genes en and wg, and on the analysis of sensory structures in gap gene mutants, it was suggested that the Drosophila pregnathal head consisted of four segments, each contributing to the brain (Schmidt-Ott et al., 1994; Schmidt-Ott et al., 1995; Schmidt-Ott and Technau, 1992). However, a detailed description of related brain neuromeres was still lacking. In order to identify positional cues and segmental boundaries during early brain development, we analysed the expression of five different segment polarity genes (en, wg, hh, mirr and gsb-d) and three DV patterning genes (msh, ind and vnd) in the procephalic neurogenic region of the ectoderm, as well as in the entire population of brain NBs derived from this region. We focused our analysis on the developmental stages 9-11 for the following reasons: (1) the complex morphogenetic reorganization during the process of head involution has not yet taken place; (2) late stage 11 represents a phylotypic stage at which the head pattern is most clearly displayed (Jürgens and Hartenstein, 1993) and the full complement of brain NBs has formed (Urbach et al., 2003), except the primordia of the optic lobes (which develop after stage 11 and fuse secondarily with the brain) (Green et al., 1993); (3) it is possible to work on the level of identified brain NB; and (4) it is also possible to correlate gene expression in the outer ectoderm and in the evolving NBs.

Our data clearly support the view that the pregnathal head consists of four segments (antennal, intercalary, ocular and labral). Furthermore, we were able to attribute to each of the four pregnathal head segments a corresponding neuromere. All segment polarity genes are segmentally expressed in the pNR as well as in brain NBs, except mirr, the segmental expression of which is not overt. wg and gsb-d are partly overlapping, and are expressed anterior to the respective en domains, which are colocalized with hh. The expression of these genes is either mainly confined to intermediate and dorsal regions of the antennal and ocular segment (in case of en, wg and gsb-d) or is at least stronger (hh) in these parts of the pNR. Consequently, with regard to segment polarity genes there is a clear segmental demarcation, which is limited to intermediate and dorsal parts of the respective neuromeres, but it remains unclear in their ventral parts (except in the tritocerebrum). Surprisingly, we find that the DV patterning genes vnd and msh endorse a separation of brain neuromeres in AP axis. As outlined above, vnd expression demarcates the ventral part of the posterior border of the tritocerebrum, deutocerebrum and ocular neuromere, and msh the dorsal anterior border of the deutocerebrum. Thus, based on the expression of segment polarity genes (en/inv, hh) and DV patterning genes (vnd, msh) we provide for the first time a reconstruction of segmental boundaries in the developing brain on the level of identified cells (Fig. 4).

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The protocerebrum is formed by the ocular segment and the posterior compartment of the labral segment

The segmental organization of the anterior head, in particular the origin of the labrum, the existence of a corresponding segment and its position at the anterior pole, are central issues of a long-lasting debate concerning head segmentation (e.g. Boyan et al., 2002; Haas et al., 2001; Jürgens and Hartenstein, 1993; Rogers and Kaufman, 1996; Schmidt-Ott et al., 1994; Scholz, 1998) (reviewed by Rempel, 1975). Consequently, the segmental origin of the protocerebrum, the largest and most anterior portion of the brain, has been a matter of debate and there is disagreement about whether it can be assigned to the labral and/or the ocular segment (equivalent to the acron).

en expression in the en dh has been attributed to the labral segment (Schmidt-Ott and Technau, 1992), the existence of which is further substantiated by PNS phenotypes in head gap mutants (Schmidt-Ott et al., 1994). We identify about 10 NBs that derive from this domain and weakly express en. Immediately anterior to the en dh, within the clypeolabral ectoderm, we find the genes wg (see also Schmidt-Ott and Technau, 1992), gsb-d, lbe and slp1 (see Urbach and Technau, 2003) to be expressed, but we observed that these domains do not contribute to the brain. The spatial pattern of expression of these genes confirms the following: the anteroposterior orientation of a labral segment, as proposed by Schmidt-Ott and Technau (Schmidt-Ott and Technau, 1992); and a parasegmental character of the border between the en dh and the labral wg domain, supporting the view that the en dh is the en-expressing part of the labral segment. We therefore conclude that the protocerebrum consists of two neuromeres, a large ocular neuromere (comprising more than 60 NBs) and a smaller labral neuromere (comprising about 10 NBs). As en expression delimits the posterior compartment of each segment (Kornberg et al., 1985), the labral neuromere appears to be confined to the posterior compartment.

The protocerebrum develops prominent neuropile structures such as the central complex and the mushroom bodies (Hanesch et al., 1989; Strausfeld, 1976). On comparative morphological grounds, the protocerebrum in arthropods has been subdivided into the archicerebrum and proscererebrum. Accordingly, the archicerebrum, which bears the optic lobes and mushroom bodies, belongs to the acron (or ocular segment) (Schmidt-Ott and Technau, 1992), and the proscererebrum, which comprises the remainder of the protocerebrum (including the central complex and the neurosecretory cells of the pars intercerebralis) belongs to the labral segment (Larink, 1970; Malzacher, 1968; Scholl, 1969) (for a review, see Rempel, 1975). We identified the progenitor cells of the mushroom bodies to be part of the ocular neuromere (R.U. and G.M.T., unpublished), supporting the view that the mushroom bodies are indeed neuropil structures of the ocular segment or archicerebrum. Consequently, the identified labral NBs would be progenitors of neurones of the pars intercerebralis. This appears likely because the en dh during further embryogenesis becomes displaced in a brain region corresponding to the pars intercerebralis of postembryonic stages (Fig. 2M). In Drosophila, little is known about the embryonic origin of the central complex. In the grasshopper, it was recently documented that NBs in the pars intercerebralis contribute neurones to the central complex (Boyan and Williams, 1997). Taking into consideration that the identified labral NBs presumably represent the progenitors of cells of the pars intercerebralis and that the fundamental ‘bauplan’ of the brain is believed to be conserved among insects (Boyan et al., 1993; Nassif et al., 1998), we suggest that, in Drosophila progeny cells of labral NBs participate in the formation of the central complex.

The segmental character of the tritocerebrum and deutocerebrum is more conserved than that of the ocular and labral neuromere

In the trunk, the neuroectoderm and NB pattern of each hemisegment is subdivided by the activity of segment polarity genes into transverse rows and by the activity of DV patterning genes into longitudinal columns (for a review, see Skeath, 1999). We find that this orthogonal expression of segment polarity and DV patterning genes is principally conserved in the posterior part of the prepupal head neuroectoderm and corresponding regions of the early brain, but becomes obscure towards anterior sites. The intercalary neuroectoderm and neuromere are subdivided by en, hh, wg and gsb-d expression into transverse-like rows and by msh, ind and vnd into longitudinal columns. Analysis of other genes that are segmentally expressed in the trunk CNS, e.g. slp1 (Bhat et al., 2000; Cadigan et al., 1994a; Cadigan et al., 1994b), ems (Hartmann et al., 2000) and lbe (R.U. and G.M.T., unpublished), provides further support for the notion that the tritocerebrum behaves like a reduced trunk neuromere (see Urbach and Technau, 2003). Similarly, this orthogonal pattern of segment polarity and DV patterning gene expression appears to be essentially retained in the antennal neuroectoderm and deutocerebrum. However, it appears less conserved compared with the tritocerebrum because en, wg and gsb-d (and slp1) expression is confined to intermediate/dorsal sites, ind is restricted to one NB and vnd is only transiently expressed. The orthogonal expression pattern of both gene groups is to a minor extent, if at all, conserved in the posterior half of the ocular neuromere. Owing to the lack of msh expression, a dorsoventral polarity is less obvious and most ocular NBs do not express any DV patterning gene. Finally, conservation of this pattern is not evident in the labral segment. Although some segment polarity genes are expressed in the labral ectoderm, expression of DV patterning genes is missing (except for the two vnd-positive NBs, Pavl and Pcv4, at the border to the ocular neuromere).

In this context, it is interesting to note that the head has been claimed to be composed of two distinct domains, an anterior terminal domain and a segmented region (Finkelstein and Perrimon, 1991). Both domains require high levels of Bicoid protein as an anterior determinant (Driever and Nüsslein-Volhard, 1988; Struhl et al., 1989), but the anterior terminal domain, which encompasses the labral segment and the acron (which is equivalent to the ocular segment) (Schmidt-Ott and Technau, 1992), is primarily specified by a signalling pathway mediated by the receptor tyrosine kinase TORSO (Klingler et al., 1988; Sprenger and Nüsslein-Volhard, 1992). Zygotic target genes which become activated by this signalling pathway (reviewed by Perrimon and Desplan, 1994) are the gap genes hkb and toll (Brönnner et al., 1994; Pignoni et al., 1990). For toll, it has been shown that (part of) its anterior, blastodermal expression is necessary for the development of the protocerebrum, which is missing in toll mutants (Pignoni et al., 1990; Rudolph et al., 1997; Strecker et al., 1988). toll represses
Fig. 5. Comparison of expression domains of DV patterning genes in the embryonic Drosophila and mouse CNS. (A,B) DV gene expression (as indicated by colour code) in the embryonic CNS of Drosophila at developmental stage 11 (A; compare Fig. 3) and mouse at ~10 days after gestation (B). Note, that anteriorly, the extent of expression is specific for each gene. Regional variabilities in the DV expansion of the respective expression domains is neglected. Mouse expression data are from Shimamura et al. (Shimamura et al., 1995) (Nkx-2.2), Valerius et al. (Valerius et al., 1995) (Gsh-1) and Shimeld et al. (Shimeld et al., 1996) (Msx-3). MD, MX, LA indicate mandibular, maxillary and labial neuromer; rh1-8, rhombomeres 1-8, respectively.

For a part of the segmented head (mandibular, intercalary and antennal) it was proposed that a combinatorial expression of the cephalic gap genes otd, ems and buttonhead (Finkelstein and Perrimon, 1990; Wimmer et al., 1993) mediates metamization by acting directly on segment polarity genes, thereby omitting the intermediate function of pair rule genes (Cohen and Jürgens, 1990) (for a review, see Finkelstein and Perrimon, 1991). More recent data indicate that, in the segmental patterning of this head region, other (intermediate) regulators are involved. One of these is collier, which is already expressed in the blastoderm and is required for the formation of the intercalary segment. It is controlled by the combined activity of ems and buttonhead, and the pair rule gene even skipped, thus integrating inputs from both the head and trunk segmentation system (Crozatier et al., 1996; Crozatier et al., 1999). Such factors might help to explain that trunk specific segmental characteristics are more conserved in the intercalary and antennal neuroectoderm and NBs, when compared to the ocular and labral neuroectoderm and NBs.

Comparison of DV patterning gene expression in the Drosophila and vertebrate brain

In Drosophila the DV patterning genes subdivide the trunk neuroectoderm into longitudinal columns (for a review, see Cornell and Ohlen, 2000; Skeath, 1999); vnd is required for the specification of the ventral neuroectodermal column and NBs (Chu et al., 1998; Jimenez et al., 1995; McDonald et al., 1998; Mellerick and Nirenberg, 1995), ind and msh have analogous functions in the intermediate and dorsal neuroectodermal columns and NBs, respectively (D’Alessio and Frasch, 1996; Isshiki et al., 1997; Weiss et al., 1998). Remarkably, homologous genes are found to be expressed in the vertebrate neural plate and subsequently in the neural tube (Fig. 5). In the neural tube the order of expression along the DV axis is analogous to that of Drosophila: like vnd, the vertebrate homologs of the Nkx family are expressed in the ventral region; the ind homologs, Gsh-1/2, are expressed in the intermediate region; and the msh homologs, Msx-1/2/3, are expressed in the dorsal region of the neural tube (for a review, see Arendt and Nübler-Jung, 1999; Cornell and Ohlen, 2000).

As already discussed, we find these DV patterning genes to be expressed in the procephalic neuroectoderm and developing brain. Furthermore, we observe that, anteriorly, the extent of expression is specific for each gene: msh is confined to more posterior regions, and vnd expression extends into anterior regions of the brain. Moreover, the expression border of msh and vnd coincide with neuromeric borders. A comparison of the anteroposterior sequence of DV patterning gene expression in the early brain of Drosophila, with that published for the early mouse brain, reveals striking similarities (Fig. 5). Msx3, which presumably represents the ancestral msh/Msx gene, becomes restricted to the dorsal neural tube during later development (in contrast to Msx1/2) (Catron et al., 1996; Shimeld et al., 1996; Wang et al., 1996). The anterior border of the Msx3 domain is positioned within the rostral region of the dorsal rhombencephalon (Wang et al., 1996), thus showing the shortest rostral extension of all vertebrate DV patterning genes. This displays analogy to msh, the expression domain of which coincides with the anterior border of the dorsal deutocerebrum, thus representing the shortest anterior extension of DV patterning genes in Drosophila. Mouse Nkx2.2 extends ventrally into the most rostral areas of the forebrain (Price et al., 1992; Shimamura et al., 1995). vnd is
expressed ventrally in anterior parts of the ocular and labral protocerebrum. Thus, the expression of the respective homologs in both species displays the most anterior extension among DV patterning genes. Moreover, Nkx2.2 expression in the mouse forebrain suggests that Nkx2.2 may be involved in specifying diencephalic neuromeric boundaries (Price et al., 1992). Similarly, in Drosophila, dorsal expansions of the vnd domain appear to correspond to the tritocerebral and deutocerebral neuromeric boundaries.

Furthermore, Drosophila ind and its mouse homologue Gsh1 show similarities in their expression in the early brain (Fig. 5). In the posterior parts of the Drosophila brain, ind is expressed in intermediate positions between vnd and msh. Likewise, in the posterior part of the mouse brain, Gsh1 appears to be expressed in intermediate positions [see Fig. 4 by Valerius et al. (Valerius et al., 1995),] dorsally to Nkx2.2 [for expression of Nkx2.2; see Fig. 3 by Shimamura et al. (Shimamura et al., 1995),] and in the hindbrain ventrally to Msx3 [see Fig. 4 by Shimeld et al. (Shimeld et al., 1996),] Gsh1 has been shown to be expressed in discrete domains within the mouse hindbrain, midbrain (mesencephalon) and the most anterior domain in the posterior forebrain (diencephalon) (Valerius et al., 1995). Correspondingly, in Drosophila we find ind expression in restricted domains within the gnathocerebrum (R.U. and G.M.T., unpublished), the tritocerebrum, deutocerebrum and ocellar part of the protocerebrum, demonstrating that the anteriormost extension of ind (and Gsh1) expression lies between that of msh and vnd.

Taken together, considering these similarities, we suggest that in the Drosophila and vertebrate early brain the expression of DV patterning genes is to some extent conserved, both along the DV axis (as suggested for the truncal parts of the Drosophila and mouse CNS) and along the AP axis. Furthermore, in Drosophila we observed that large parts of the anterodorsal procephalic neuroectoderm and NBs (more than 50% of all identified brain NBs) lack DV patterning gene expression. Likewise, in the vertebrate neural tube, gaps between the expression domains of DV patterning genes have been described, raising the possibility that other genes might fill in these gaps (Weiss et al., 1998). How DV fate is specified in the anterior and dorsal part of the Drosophila procephalic neuroectoderm, and if other genes are involved, remains to be clarified.

We are grateful to Andreas Prokop, Joachim Urban and Ana Rogulja-Ortmann for critical comments on the manuscript; and to Fernando Jiménez, Bob Holmgren, Krzysztof Jagla, Jym Mohler, Akinao Nose, Matthew Scott, Harald Vässin, Helen McNeill, Yuh-Nung Yan and the Bloomington stock center for providing antibodies, cDNA and fly stocks. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to G.M.T.

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