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 fulfil 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. 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 homebox, 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
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 presegmental 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 neuromeres, 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 dorsoventral 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 presegmental *Drosophila* head is composed of four segments, and we now attribute to each of the four presegmental segments a corresponding neuromere. Furthermore, we provide evidence that the protocerebrum consists of two neuromeres, which derive from the ocular and labral segment. The segmental character of these neuromeres is less conserved compared with the trito- and deutocerebrum, deriving from the intercalary and antennal segment. Finally, we discuss similarities in the expression of dorsoventral 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* (tyXho25) (Hama et al., 1990), *hedgehog-lacZ* (16E) (Mohler et al. 1995) (kindly provided by J. Mohler), *mirror-lacZ* (Broadus et al., 1995; McNeill et al., 1997) (kindly provided by H. McNeill and M. Simon), *muscle segment homeobox-lacZ* (H96) (Isshiki 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-Invected (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 neuromere along the AP axis (Bhat, 1996; Broads et al., 1995; Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Zhang et al., 1994). In the presegmental 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 presegmental 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 neuromeres, and to map the position of their boundaries, we analysed the expression of segment polarity genes, including *wingless*, *hedgehog*, *gooseberry-distal*, *engrailed*, *invected* 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* 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 presegmental head (neuroectoderm: Fig. 1B,C,E,F; Fig. 2A,C,E,I,K,L) (NBs: Fig. 1A,D; Fig.
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2B,D,F,I,M) we find en expression as follows: from late stage 8
in the posterior ectoderm of the antennal segment (en antennal
stripe; en as) from which four deutocerebral NBs (Dv8, Dd5,
Dd9, Dd13) delaminate; from stage 9 in a small ectodermal
domain in the posterior part of the ocular segment, the en head
spot (en hs), from which two protocerebral NBs (Ppd5, Ppd8)
evolve; and from stage 10 in an ectodermal stripe in the posterior
intercalary segment (en intercalary stripe; en is), which gives rise
to three to four tritocerebral NBs (Tv4, Tv5, Td3, Td5).
Furthermore, from stage 11 onwards, En is weakly detected in
the anteriormost ectoderm of the procephalon corresponding to
the region of the ‘anterior dorsal hemispheres’ (en dh) (Rogers
and Kaufman, 1996; Schmidt-Ott and Technau, 1992) (Fig. 2I-
N) (see also Urbach et al., 2003). We identified about 10 weakly
En-positive NBs, which delaminate from the
en dh (Fig. 2I,M).
Thus, consistent with earlier results (Schmidt-Ott and Technau,
1992), we find that all four pregnathal head segments contribute
to the early embryonic brain. The spatial distribution of the En-
positive NBs closely corresponds to the
en domains of their
origin in the ectoderm. This suggests they demarcate the
posterior borders of the respective brain neuromeres (Fig. 4).
In the trunk, hedgehog (hh) matches en expression (Mohler
and Vani, 1992; Tabata et al., 1992). This is also the case for the
intercalary segment in the pregnathal head ectoderm (Fig. 1C,F;
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. 2LJ). 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 deuto- and protocerebrum in the dorsal direction (Fig. 1D, Fig. 2L, Fig. 4).

From late stage 8 onwards, Wingless (Wg) protein is expressed in a neuroectodermal domain spanning a broad area
Fig. 2. Expression of segment polarity genes at embryonic stage 11 (st11). (A,C,E,G) Left half of head flat preparations double labelled for segment polarity gene expression and en-lacZ, inverted (im) or ladybird (lbe); focus is on the peripheral head ectoderm; broken line marks the ventral midline; insets depict lateral views of stage 11 whole-mount preparations. (B,D,F,H) Close-ups of regions indicated in A,C,E,G by black frames; focus is on the level of NBs; immunopositive NBs are indicated by black inscription, immunonegative NBs are indicated by black inscription. (A,B) Only part of the antennal/ocular hh-lacZ domain co-expresses imv. Note the strong hh-lacZ signal in the dorsal Pdp5, Pdp8, Pdp10, Pdp11, Pdp15 and Pdp16. (C) gsb-d is downregulated in the ocular neuroectoderm, and is detectable in four tritocerebral NBs deriving from the gsb-d intercalary stripe, and in three deutocerebral NBs deriving from the gsb-d antennal stripe (D,LJ). (E,F) mirr-lacZ expression shows no segmental pattern and is mainly limited to the ventral part of the PNR and corresponding NBs. (G) lbe is segmentally expressed in the procephalic neuroectoderm [arrows; for details, see Urbach and Technau (Urbach and Technau, 2003)], where it is co-expressed with wg, except in the wg hb and wg fg. (H) lbe is co-expressed with wg in Dd7 but not in the ocular Ppd3 and Pcv8. (I) Segment polarity gene expression in identified brain NBs at stage 11: nomenclature of brain NB has been described previously (Urbach et al., 2003). Colour intensity reflects weak (-) and strong (+) expression levels of imv, hh-lacZ and wg. Stars indicate mandibular NBs. (J.K) Segment polarity gene expression in the peripheral procephalic ectoderm. (L-N) inv expression in the dorsal hemispheres (dh). (L) Lateral view of late stage 11 head, showing a faint inv expression in the dh. Note that en expression in the dh is not detected using en-lacZ and was only observed with anti-Inv antibodies from late stage 11 onwards. (M) Left half of a head flat preparation. The dh comprises about 10 Inv-positive NBs (as depicted in I). (N) inv expression in the brain and sub-oesophageal ganglion (SOG) of a stage 10 embryo (horizontal view). Note that Inv staining in the dh corresponds to the pars intercerebralis of postembryonic stages. The secondary head spot (shs) marks a small group of cells which secondarily separates from the hs (see Schmidt-Ott and Technau, 1992). 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; dh, en expression in the dorsal hemispheres; hs, en head spot; is, en intercalary stripe; shs, en secondary head spot; gsb-d as, gsb-d antennal stripe; gsb-d is, gsb-d intercalary stripe; gsb-d lr, gsb-d labral spot; wg as, wg antennal stripe; wg fg, wg expression in the foregut; wg hb, wg ocular head blob; wg is, wg intercalary spot; wg lr, wg labral spot.

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. 2GJ). 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. 2GJ), 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. 2HI), 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 pre-embryonic 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. 2CJ). 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,F1,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. 2L), 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 \textit{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 \textit{ventral nervous system defective} (\textit{vnd}), \textit{intermediate neuroblast defective} (\textit{ind}) and \textit{muscle segment homeobox} (\textit{msh}) have been shown to confer positional information to the truncal neuroectoderm, which also contributes to the specification of NBs (reviewed by Skeath,
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**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 *vnd* domain; the emerging Dd1 does not express *vnd*. (B,D) Stage 10; *vnd* is already downregulated in part of the ventral antennal ectoderm 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,I,K) 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. (H,I,L) Close-ups of regions indicated in G,I,K 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 deutocerebral and ocular protocerebral NBs (H). (LJ) 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 ectoderm (K) and NBs (L); note the dorsally directed extension of *vnd* domains in the antennal 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 deutocerebral 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, 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 Nirengberg, 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 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).
anlagen. Anlagen of the brain, including the deutocerebral NBs (Dv2 and Dv4), are shown. Red lines indicate the borders between the tritocerebrum (Tc; comprising about 13 NBs), the deutocerebrum (Dc; comprising about 21 NBs), and the clypeolabrum (CL; comprising about 21 NBs). The expression of msh is first detected at stage 7, and its expression is dynamic and from stage 9 onwards is downregulated in parts of the antennal neuroectoderm and deutocerebral NBs (see vnd section).

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 (Ishikii et al., 1997), it is not expressed in the preantennal head ectoderm (Fig. 3C-H).

2. ind expression is continuous in the trunk, but forms three segmental patches in the procephalon. ind expression in the segmental patches 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 segments 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,I), 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), 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).
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, Ppv1, Ppv2* and *Ppv3* would demarcate the ventral part of this border. The intermediate part is defined by the *en/hh-co-expressing Ppd5* and *Ppd8*, 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 larval segment. The existence of a larval 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 phyloptic 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).
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; 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).

\[ \text{en} \] expression in the \[ \text{en} \] 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 \[ \text{en} \]. Immediately anterior to the \[ \text{en} \] dh, within the clypeolabral ectoderm, we find the genes \[ \text{wg} \] (see also Schmidt-Ott and Technau, 1992), \[ \text{gsb-d} \], \[ \text{lbe} \] and \[ \text{slp1} \] (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 \[ \text{en} \] dh and the labral \[ \text{wg} \] domain, supporting the view that the \[ \text{en} \] dh is the \[ \text{en} \]-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 \[ \text{en} \] 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 prosocerebrum. 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 prosocerebrum, which comprises the remainder of the protocerebrum (including the central complex and the neurosecretory cells of the pars intercerebralis) belongs to the labral segment (Larkin, 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 \[ \text{en} \] dh during further embryogenesis becomes displaced in a brain region corresponding to the pars intercerebralis of postembryonic stages (Fig. 2M). In \[ \text{Drosophila} \], 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 \[ \text{Drosophila} \] 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 pregnathal head neuroectoderm and corresponding regions of the early brain, but becomes obscure towards anterior sites. The intercalary neuroectoderm and neuromere are subdivided by \[ \text{en} \], \[ \text{hh} \], \[ \text{wg} \] and \[ \text{gsb-d} \] expression into transverse-like rows and by \[ \text{msb} \], \[ \text{ind} \] and \[ \text{vnd} \] into longitudinal columns. Analysis of other genes that are segmentally expressed in the trunk CNS, e.g. \[ \text{slp1} \] (Bhat et al., 2000; Cadigan et al., 1994a; Cadigan et al., 1994b), \[ \text{ems} \] (Hartmann et al., 2000) and \[ \text{lbe} \] (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 \[ \text{en} \], \[ \text{wg} \] and \[ \text{gsb-d} \] (and \[ \text{slp1} \]) expression is confined to intermediate/dorsal sites, \[ \text{ind} \] is restricted to one NB and \[ \text{vnd} \] 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 \[ \text{msb} \] 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 \[ \text{vnd} \]-positive NBs, \[ \text{Pav1} \] and \[ \text{Pcv4} \], 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 \[ \text{hhb} \] and \[ \text{tll} \] (Brönner et al., 1994; Pignoni et al., 1990). For \[ \text{tll} \], it has been shown that (part of) its anterior, blastoderma expression is necessary for the development of the protocerebrum, which is missing in \[ \text{tll} \] mutants (Pignoni et al., 1990; Rudolph et al., 1997; Strecker et al., 1988). \[ \text{tll} \] represses
hb and fj2 and may thus function in the head as an ‘anti-
segmentation’ gene (Reinitz and Levine, 1990). We find that
tll expression, which covers the ocular and labral
neuroectoderm (the latter of which coincides with the region of
the en dh) and emerging NBs (Urbach and Technau, 2003)
(see also Rudolph et al., 1997), closely corresponds to that part
of the early brain where segmental features are largely obscure.
A coordinated, orthogonal expression of segment polarity
and DV patterning genes within the ocular and labral
neuroectoderm is not obvious, and the existence of putative
serially homologous NBs in those regions of the brain is less
evident (Urbach and Technau, 2003). This implies that tll might
be a component crucial for the suppression of segmental
characteristics in the ocular and labral neuromere.
Furthermore, crossregulatory interactions among the segment
polarity genes in the pregnathal head differ from those in the
trunk and are unique for each pregnathal segment (Gallitano-
Mendel and Finkelstein, 1997).

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
metamerization 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 neuroectodermic boundaries (Price et al., 1992). Similarly, in *Drosophila*, dorsal expansions of the *vnd* domain appear to correspond to the tritocerebral and deutocerebral neuroectodermic 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 *Msh*3 [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 anterior/most 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.

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