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The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in *Drosophila*

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SUMMARY

In the *Drosophila* embryo, studies on CNS development have so far mainly focused on the relatively simply structured ventral nerve cord. In the trunk, proneural genes become expressed in small cell clusters at specific positions of the ventral neuroectoderm. A lateral inhibition process mediated by the neurogenic genes ensures that only one cell within each proneural cluster delaminates as a neural stem cell (neuroblast). Thus, a fixed number of neuroblasts is formed, according to a stereotypical spatiotemporal and segmentally repeated pattern, each subsequently generating a specific cell lineage. Owing to higher complexity and hidden segmental organisation, the mechanisms underlying the development of the brain are much less understood. In order to pave the way towards gaining deeper insight into these mechanisms, we have undertaken a comprehensive survey of early brain development until embryonic stage 11, when all brain neuroblasts have formed. We describe the complete spatiotemporal pattern of formation of about 100 brain neuroblasts on either side building the trito-, deuto- and protocerebrum. Using 4D-microscopy, we have uncovered various modes of neuroblast formation that are related to specific mitotic domains of the procephalic neuroectoderm. Furthermore, a detailed description is provided of the dynamic expression patterns of proneural genes (*achaete*, *scute*, *lethal of scute*, *atonal*) in the procephalic neuroectoderm and the individual neuroblasts. Finally, we present direct evidence that, in contrast to the trunk, adjacent cells within specific domains of the procephalic neuroectoderm develop as neuroblasts, indicating that mechanisms controlling neuroblast formation differ between head and trunk.

Key words: CNS, Brain development, Neuroectoderm, Neuroblasts, Proneural genes, Mitotic domains, Lateral inhibition, *Drosophila*

INTRODUCTION

Uncovering the mechanisms that lead to pattern and cell diversity in the central nervous system is one of the major challenges in developmental biology. The established molecular and genetic tools as well as micromanipulation techniques have made *Drosophila* a suitable model organism to study these mechanisms. The embryonic central nervous system (CNS) in insects develops from a bilateral, two-dimensional sheet of cells, the neuroectoderm, from which multipotent stem cells, the neuroblasts (NBs), delaminate (Wheeler, 1891). The NBs, which form the truncal CNS (ventral nerve cord and suboesophageal ganglion), delaminate from the ventral neurogenic region, whereas the NBs that form the brain derive from the procephalic neurogenic region (Poulson, 1950). Owing to its much simpler organization, studies on mechanisms that control early neurogenesis in *Drosophila* have mainly focused on the ventral nerve cord (VNC). Through the expression of proneural genes of the *Achaete-Scute-Complex* at precise locations, groups of neuroectodermal cells, called proneural clusters, acquire the potential to become NBs (Cabrera, 1987; Jimenez and Campos-Ortega, 1990; Skeath et al., 1992). Cell-cell interactions, which are mediated by the neurogenic genes, ensure that in each proneural cluster only a single cell with the highest level of proneural gene expression adopts a NB fate, while the others remain in the periphery to develop as epidermoblasts (reviewed by Campos-Ortega, 1995). The singling out of NBs follows a stereotypical spatial and temporal pattern (Doe, 1992; Hartenstein and Campos-Ortega, 1984). Upon delamination, NBs typically undergo repeated asymmetric divisions, budding off smaller ganglion mother cells, which divide once to produce neurones and/or glial cells (reviewed by Goodman and Doe, 1993). In this way, each NB produces a specific cell lineage (Bossing et al., 1996; Schmidt et al., 1997). The fate of the individual NBs depends on their position within the neuroectoderm (reviewed by Bhat, 1999; Skeath, 1999), time of delamination (Berger et al., 2001) and the combination of genes they express (Broadus et al., 1995; Doe, 1992).

In contrast to the VNC, our understanding of brain development is still very rudimentary. Which developmental mechanisms lead to the significant differences between the specification and differentiation of structures in the brain and

VNC, as well as among regions within the brain itself? What is the evolutionary origin of brain-specific structural and functional complexity? An important basis for approaching these questions is the clarification of the composition and developmental origin of the various brain structures at the cellular level, and the identification of genes expressed in the respective structures and individual cells. The insect brain is traditionally subdivided into the tritocerebrum, deutocerebrum and protocerebrum (Bullock and Horridge, 1965; Hanström, 1928), which derive from the intercalary, antennal and ocular/labral head segments, respectively (e.g. Hirth et al., 1995; Rempel, 1975; Schmidt-Ott and Technau, 1992; Younossi-Hartenstein et al., 1996). In the adult fly brain, highly organized neuropil structures have been described, such as the mushroom bodies, central complex, optic lobes, antennal lobes and other specialized neuropils and major fibre tracts, which have no counterparts in the VNC (e.g. Hanesch et al., 1989; Power, 1943; Strausfeld, 1976). Main structural characteristics of the bauplan of the adult brain are already laid down during embryogenesis (Hassan et al., 2000; Kurusu et al., 2000; Nassif et al., 1998; Noveen et al., 2000), but it is largely unclear how these structures evolve from the neuroectoderm and corresponding NBs.

In this and the accompanying papers (Urbach and Technau, 2003a; Urbach and Technau, 2003b) we have undertaken a comprehensive survey of *Drosophila* early brain development (stages 8-11), including the pattern of NB formation, the segmental organization of the brain, and the genes expressed in the procephalic neuroectoderm as well as in the individual NBs. We provide a detailed description of the spatiotemporal development of the entire population of about 100 NBs forming the trito-, deuto- and protocerebrum (including glial and sensory precursors), and assign a systematic nomenclature to the individual NBs. We describe in detail the expression patterns of proneural genes of the *Achaete-Scute-Complex* and *atonal* in the procephalic neurogenic ectoderm and in the brain NBs. We show that at least four of the procephalic mitotic domains described by Foe (Foe, 1989) contribute to the embryonic brain. Using 4D microscopy we demonstrate that brain NB formation is achieved in distinct ways related to the respective mitotic domain. Furthermore, we show that in a central part of the procephalic neuroectoderm several NBs originate from adjacent cells in contrast to the trunk where only one cell of each proneural cluster adopts a NB fate. This and the patterns of proneural gene expression indicate that modes of NB formation differ between head and trunk.

MATERIALS AND METHODS

Drosophila strains

The following fly strains were used: Oregon R (wild type), *engrailed-lacZ* (ryXho25) (Hama et al., 1990), *seven up-lacZ* (H162) (Mlodzik et al., 1990), *A37-lacZ* (Ghysen and O'Kane, 1989) and *scute-lacZ* (Martinez and Modolell, 1991) (kindly provided by J. Modolell).

Staging of embryos

Staging of the embryos was carried out according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997); additionally, we used the well-characterized trunk NB pattern (Doe, 1992) as a further reference system for staging.

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), mouse-anti-Achaete (mAb 984A11C1) (1:3) (Skeath and Carroll, 1992) (kindly provided by J. Skeath), rabbit-anti-Atonal (1:5000) (Jarman et al., 1993) (kindly provided by A. Jarman), anti-DIG-AP (1:1000, Roche), rabbit-anti-Deadpan (1:300) (Bier et al., 1992) (kindly provided by H. Vässin), mouse-anti-Invected (4D9) (1:4) (Patel et al., 1989) (Developmental Studies Hybridoma Bank), mouse-anti-β-galactosidase (1:500, Promega), rabbit-anti-β-galactosidase (1:2500, Cappel), mouse-anti-Ladybird early (1:2) (Jagla et al., 1997) (kindly provided by K. Jagla), rat-anti-Lethal of scute (1:500) (Martin-Bermudo et al., 1991) (kindly provided by J. Skeath), rabbit-anti-Repo (1:100) (Halter et al., 1995) and mouse-anti-alpha-Tubulin (1:100, Sigma). 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 were diluted 1:500.

Whole-mount in situ hybridization

DIG labelled *glial cells missing* (*gcm*) RNA probe (kindly provided by Y. Hotta) was synthesized with T7 RNA polymerase and *Xba*I linearised pBlue-gcm as a template according to the manufacturer's protocol (Roche). The hybridization of embryos was performed as described previously (Plickert et al., 1997; Tautz and Pfeifle, 1989).

Flat preparation

The abdomen and yolk of stained embryos were removed in 70% glycerol in 0.1 M PBS, and the head capsule was opened along the dorsal midline. Each dissected embryo was placed in a small drop of 80% glycerol in between two coverslips (upper coverslip 18×18 mm, lower 60×22 mm), carefully flattened and sealed with nail-polish. Flat preparations embedded in this way can be viewed from both sides, and allow for significantly better microscopic resolution compared with wholemounts (compare Fig. 3C,E,G with 3D,F,G).

Documentation

Embryos were viewed under a Zeiss Axioplan equipped with Nomarski optics, 40×, 63× and 100× oil immersion objectives and a CCD camera (Contron progress 3012). Different focal planes were combined using Adobe Photoshop 6.0. Semi-schematic presentations are based on camera lucida drawings.

4D microscopy

Wild-type eggs were collected and mechanically dechorionated at the blastoderm stage. Single embryos were fixed to the surface of a coverslip (22×60 mm, coated with glue) in an anterolateral orientation, so that the main part of the procephalic ectoderm becomes attached to the coverslip in one focal plane. Each embryo was covered with about 5 μl fluorocarbon oil (10S). The coverslip with the mounted embryos was transferred onto a second coverslip (22×60 mm; carrying thin distance brackets at both ends) so that the embryos are oriented upside down between both coverslips, allowing subsequent examination under an upright microscope.

For in vivo tracing and documentation of early embryonic development of the procephalic region (at about 25°C) 4D microscopy was applied. The basics of this technique to record a three dimensional time-lapse movie are described by (Schnabel et al., 1997). The instrumentation was now improved (R.S., unpublished), and allows images of very high quality to be stored on the computer. The temperature-controlled stage of a Zeiss Axioplan microscope was moved by a piezo focusing device (Physik Instrumente D-76337 Waldbronn) to record the *z*-series (<50 focal levels, typically 1 μm per focal level; depending on the number of focal levels, recording is repeated every 30 to 60 seconds). The analogue pictures are collected with a Hamamatsu Newvicon camera, digitised with an Inspecta-3

frame grabber (Mikroton, D-85386 Eching) and finally compressed to 40 kb per picture with a wavelet function (Lurawave, D-10587 Berlin). The microscope and the accessories are controlled with a PC using a specially designed software (4DDM, AK Schulz and RS) programmed in C++. The 4D-records are replayed and cell positions and cleavages are documented with the database SIMIBiocell (SIMI D-85705 Unterschleißheim).

RESULTS

Identification and nomenclature of brain neuroblasts

Brain neuroblasts (NBs) were morphologically identified (using Nomarski optics) by their position (below the peripheral neuroectoderm), larger size (diameter usually >10 μm) and round shape, and by the expression of stem-cell specific markers like *deadpan* (*dpn*) (Bier et al., 1992) or *asense* (*ase*) (Brand et al., 1993). NB identities are indicated by their position relative to the cephalic furrow, invaginating foregut, dorsal and ventral midline, their relative position within the NB pattern, their time of segregation, and the expression of cell-specific markers (Fig. 3) (Urbach and Technau, 2003a; Urbach and Technau, 2003b). Following the nomenclature introduced by Younossi-Hartenstein et al. (Younossi-Hartenstein et al., 1996), brain NBs are named according to their assignment to the trito- (T), deuto- (D) and protocerebrum (P), and within the protocerebrum to an anterior (Pa), central (Pc) and posterior (Pp) group. Assignment of individual NBs to particular neuromeres is based on the reconstruction of segmental borders as detailed by Urbach and Technau (Urbach and Technau, 2003a), and the three protocerebral groups roughly reflect their origin from distinct mitotic domains (see below). Differing from the nomenclature by Younossi-Hartenstein et al. (Younossi-Hartenstein et al., 1996), we further subdivide each of the protocerebral groups, as well as the deuto- and tritocerebral neuroblasts, into a dorsal (d) and a ventral (v) subgroup based on the expression of the D/V patterning gene *vnd* (see Urbach and Technau, 2003a). Finally, within each of these subgroups, individual neuroblasts are numbered (1,2, etc.) from anterior-to-posterior and from ventral-to-dorsal sites (so that numbers reflect relative positions along the DV axis; see Fig. 1). Our nomenclature is based on the complete late stage 11 NB array, and is also used for corresponding NBs in embryos younger than late stage 11 (Fig. 2). Although for better resolution the pictures and semi-schematic NB maps shown in the following generally correspond to flat preparations, they can be also applied to the identification of NBs in whole mounts, as demonstrated in Fig. 3.

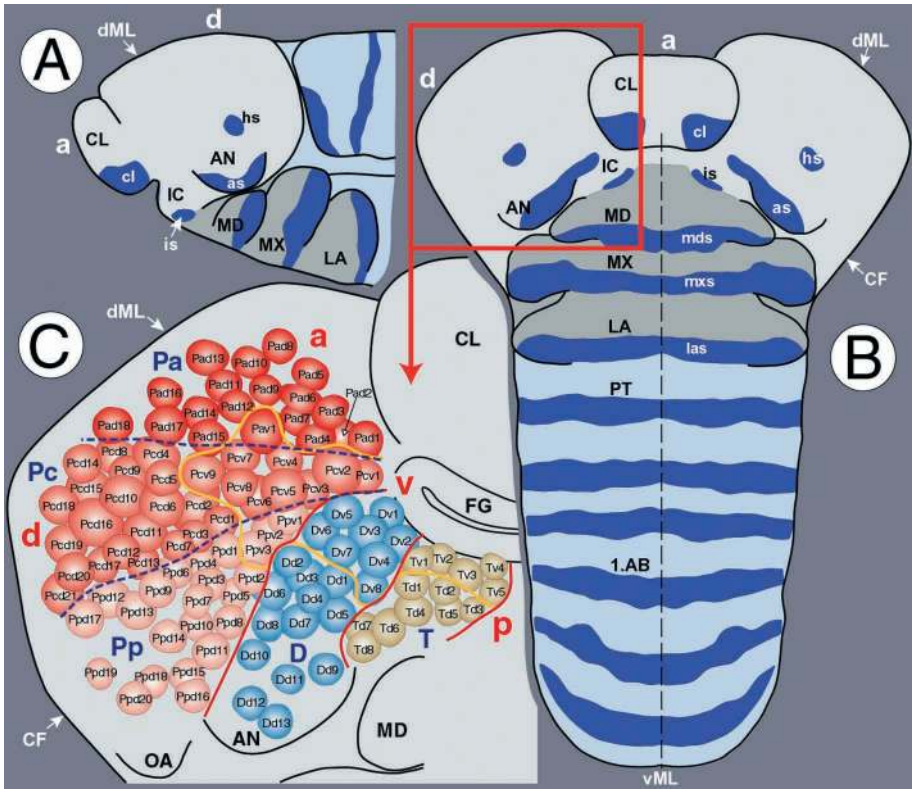


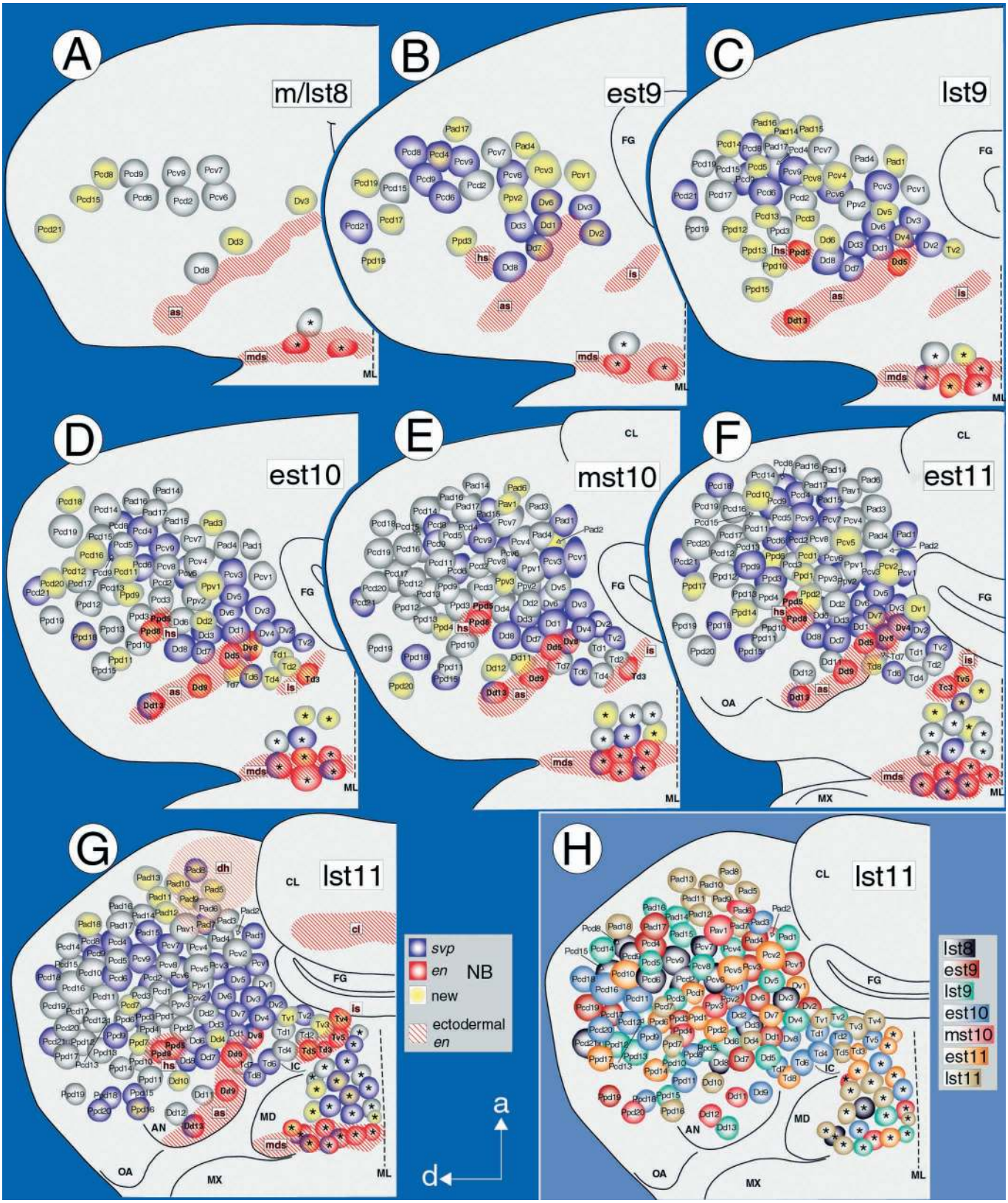
Fig. 1. Survey of the spatial organisation of the embryonic head and brain neuroblasts at stage 11. Semi-schematic drawing showing a lateral view of a (A) wholemount and (B) a ventral view of a flat preparation, in which the head capsule has been dorsally opened. The pregnathal (light grey) and gnathal (dark grey) head region is indicated in relation to the *engrailed* expression pattern (dark blue). (C) Left half of a head flat preparation, including the complete pregnathal NB pattern. NBs are named according to their assignment to the trito- (T), deuto- (D) and protocerebrum (P), based on the reconstruction of segmental borders [indicated by red lines; for details see Urbach and Technau (Urbach and Technau, 2003a)]. Protocerebral NBs are subdivided into an anterior (Pa), central (Pc) and posterior (Pp) group (indicated by blue broken lines), roughly reflecting their origin from distinct mitotic domains (see Fig. 5 and Table 1). Each of the protocerebral groups, as well as the deuto- and tritocerebral NBs, is further subdivided into a dorsal (d) and a ventral (v) subgroup (indicated by yellow line) based on *vnd* expression [except NB Dd5, which co-expresses *msh* and is therefore attributed to the dorsal deutocerebrum (Urbach and Technau, 2003a)]. Within each subgroup, NBs are numbered from anterior to posterior and from ventral to dorsal. a, d, p, v: anterior, dorsal, posterior, ventral. as, is, las, mds, mxs: antennal, intercalary, labial, mandibular and maxillary *en* stripe, respectively. cl: *en* expression in the clypeolabrum. hs: *en* head spot. AN, IC, LA, MD, MX, PT, 1.AB: antennal, intercalary, labial, mandibular, maxillary, prothoracic and first abdominal segments, respectively. CF: cephalic furrow. CL: clypeolabrum. dML: dorsal midline. FG: foregut. OA: Bolwig organ/optic lobe anlagen. vML: ventral midline.

Taken together, this nomenclature reflects topological and functional characteristics, addresses all the identified brain neuroblasts individually, is convenient for the user and is flexible enough to accommodate new data.

Procephalic neuroblasts develop in a stereotypical spatial and temporal pattern

We traced the pattern of brain NBs through the entire period of NB formation (stage 8-11) in fixed flat preparations of staged embryos. We subdivide NB formation into seven stages (Fig. 2). Some of them correspond to stages where NB patterns

have been previously described in the trunk (Broadus et al., 1995; Doe, 1992; Hartenstein et al., 1987), allowing a comparison of the development of NB patterns in the trunk and procephalon. Camera lucida drawings were prepared showing the typical arrangement of NBs at the respective stages (Fig. 2). The spatial arrangement of NBs is largely invariant. In addition, the temporal sequence of formation from the neuroectoderm follows a reproducible pattern, although the



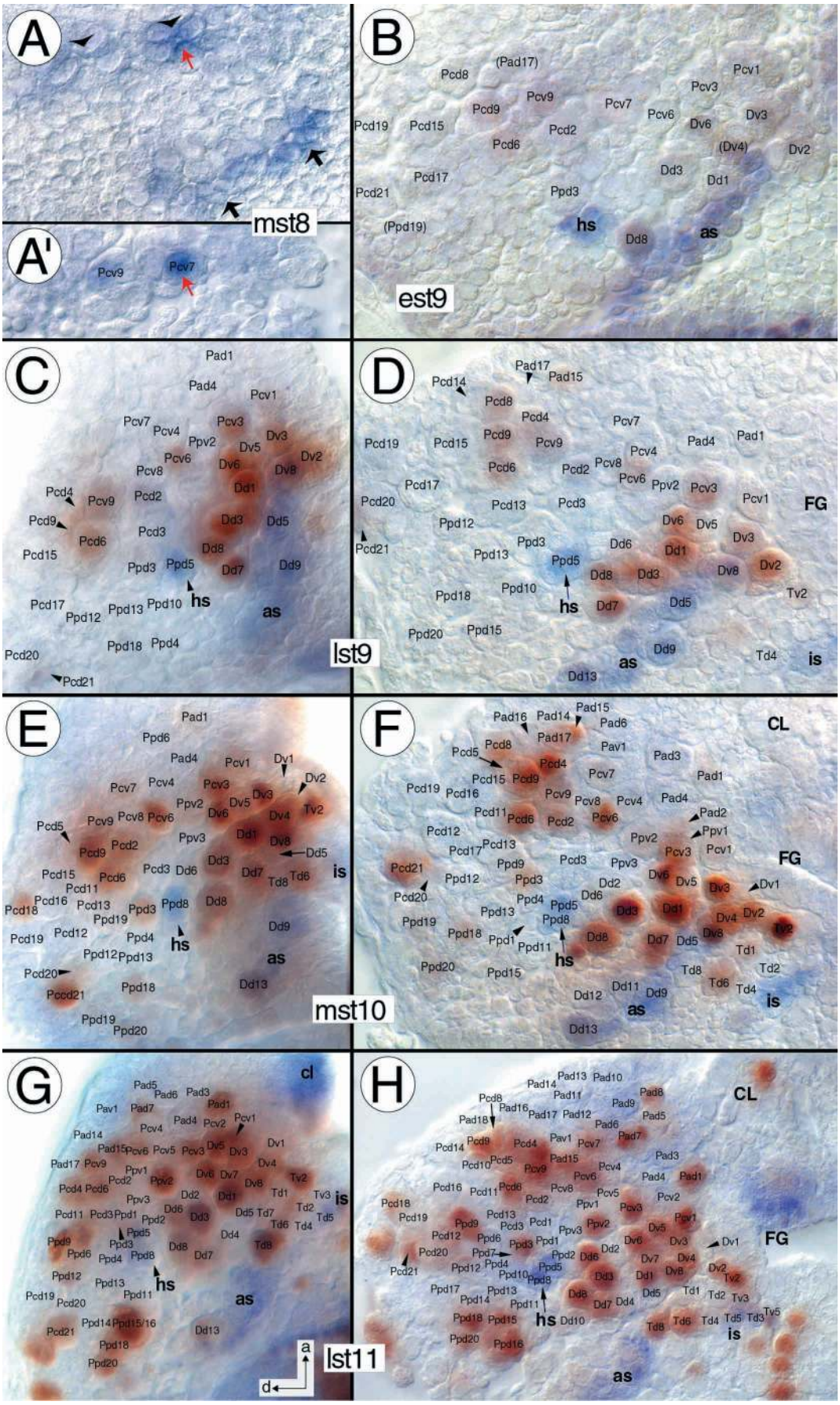
time point at which particular NBs are formed can vary to a certain degree, as was described for NBs in the trunk (Bossing et al., 1996; Schmidt et al., 1997). Intermediate brain NB patterns between the illustrated stages can therefore be observed.

The procephalon consists of four fused segments: the labral, ocular, antennal and intercalary segment (from anterior to posterior) (Schmidt-Ott et al., 1994; Schmidt-Ott and Technau, 1992). Neurogenesis in the procephalic ectoderm, as in the trunk, initiates at early stage 8. At this stage antibody staining reveals Dpn expression in neuroectodermal domains in the antennal and ocular segment (Fig. 3A). By mid-stage 8 these domains give rise to first brain NBs, which can be uniquely addressed in flat preparations by their absolute position in the overlaying procephalic neuroectoderm and relative position within the NB pattern (Fig. 2A, Fig. 3B). As the NB pattern becomes more complex in the later stages, we examined molecular markers that are expressed in subsets of brain NBs, such as *engrailed* (*en*, revealed by an *en-lacZ* line or an antibody against 4D9 recognizing the products of the closely related *en* and *invected* genes) (Coleman et al., 1987) and *seven up* (*svp*, revealed by *svp-lacZ* enhancer trap line H162) (Mlodzik et al., 1990), as well as an array of other markers (see Urbach and Technau, 2003a; Urbach and Technau, 2003b). *en* expression allows for a clear distinction of gnathal and pregnathal segments. In the pregnathal head, it is expressed in several ectodermal domains and descending NBs, thus demarcating boundaries between head segments (Schmidt-Ott and Technau, 1992) and corresponding trito-, deuto- and protocerebral neuromeres. During stages 9-11, *svp* and *en* are continuously expressed in an increasing amount of single NBs or clusters of brain NBs. Thus, Svp- and En-positive NBs present stable reference points for the identification of surrounding NBs. The onset of *svp* expression is characteristic for each NB. It is generally initiated in NBs during or shortly after formation, but in a few exceptions *svp* expression begins quite some time after formation (e.g. Pcv1 develops at early stage 9, but Svp cannot be detected before stage 10; Figs 2, 3). Additionally, the level of *svp-lacZ* expression appears to differ significantly and specifically among NBs of the same stage (e.g. at late stage 9 it is higher in Dd1 or Dv6 compared with Pcv3 or Pcv6; Fig. 3D). We find that some new NBs are added at the borders of the NB array, but that others become interspersed between existing NBs (also at later stages). This is in contrast to earlier reports suggesting that brain NBs become sequentially added only in a centrifugal way (Younossi-Hartenstein et al., 1996). Until late stage 9 in the procephalon (as in the trunk) (Doe, 1992), approximately half of the total number of brain NBs is formed, encompassing 12 deuto- and 34 protocerebral NBs (Fig. 2C, Fig. 3C,D). An orthogonal patterning of brain NBs in columns and rows, as described for the trunk (Doe, 1992; Hartenstein and Campos-Ortega, 1984), is not apparent. This is corroborated by the expression of dorsoventral patterning genes and segment polarity genes (see Urbach and Technau, 2003a).

Until late stage 11 about 106 brain NBs have formed on either side (Fig. 2G). As we do not find additional NBs to be formed during stage 12 (for NB identification see above), we conclude that by late stage 11 the pattern of embryonic brain NBs is complete (consistent with the situation in the trunk) (Doe, 1992). It comprises about 72 protocerebral, 21 deutocerebral and 13 tritocerebral NBs. Svp is reproducibly expressed in about 39 of all NBs, En is strongly expressed by about 10 NBs [Tv4, Tv5, Td3, Td5 emerging from the engrailed intercalary stripe, 'en is'; Dv8, Dd5, Dd9, Dd13 from the engrailed antennal stripe, 'en as'; Ppd5, Ppd8 from the engrailed head spot, 'en hs'; for nomenclature of *en* expression domains in the procephalic ectoderm see Schmidt-Ott and Technau (Schmidt-Ott and Technau, 1992)] and weakly by a cluster of about 10 NBs in the anteriomost part of the protocerebral primordium (Fig. 2). In the observed developmental period, the positions of brain NBs relative to each other and to the outer ectoderm (e.g. taking ectodermal *en* domains as reference points) in principal do not change, except for slight variabilities that might be due to new NBs becoming accommodated into the pattern.

Cell size varies between NBs. Apparently, most of the early NBs are larger than later developing NBs (e.g. Dd8 being formed at stage 8 is significantly larger than the adjacent Ppd5 and Ppd8, which form at late stage 9/early stage 10; Fig. 2D,E, Fig. 3E). Also in the trunk early (S1/2), NBs are generally larger than late (S4/5) NBs, and this has been shown to be correlated with a previous division of late NBs in the neuroectoderm (Bossing et al., 1996; Schmidt et al., 1997).

Fig. 2. Spatial and temporal development of the embryonic brain NB pattern. (A-G) Semi-schematic representations of ventral views on the left half of head flat preparations, double stained for *svp-lacZ* and Engrailed (*en-lacZ* or anti-Invected antibody) at (A) mid/late stage 8 (m/lst8), (B) early stage 9 (est9), (C) late stage 9 (lst9), (D) early stage 10 (est10), (E) mid stage 10 (mst10), (F) early stage 11 (est11) and (G) late stage 11 (lst11). Expression of *svp-lacZ* and *engrailed* (*en*) in NBs is indicated in blue and red, respectively; newly formed NBs at each stage are in yellow; red hatching marks *en* expression in the peripheral ectoderm. Anterior (a) is towards the top and dorsal (d) is towards the left. Note, most of the depicted stages correspond to those described as phases 'S1-S5' for the pattern of NB formation in the trunk (Doe, 1992): m/lst8 corresponds to early S1; est9 to S1; lst9 to S2; e/mst10 to S3; est11 to S4; and lst11 to S5 phase. In addition to the pregnathal segments, the evolving NB pattern is also shown for the mandibular segment (MD; asterisks indicate mandibular NBs). At early/mid stage 9 (B), when *svp* expression initiates, it is detected in a simple pattern, including about seven protocerebral and all deutocerebral NBs. At late stage 9 (C), two En-positive deutocerebral NBs (Dd5 and Dd13) derive from the *en* antennal stripe (as), and one protocerebral NB (Ppd5) from the *en* head spot (hs). New NBs form at the borders of the developing NB array, but in addition, individual NBs become integrated at various positions into the pre-existing NB pattern (D-G). By late stage 11 (G), Inv (but not *en-lacZ*) is weakly detected in the anteriormost procephalon (dh); the faint Inv expression in about 10 NBs deriving from the dh is not indicated (see Urbach and Technau, 2003a). Note that the formation of NBs in the intercalary (IC) and (anterior) mandibular segment (MD) is significantly delayed. Formation of tritocerebral NBs starts at stage 10. (H) Fully developed NB array (lst11) with the stage of formation indicated for each cell (see key). Most of the early (stage 8/early stage 9) NBs occupy central parts of the protocerebral primordium at different D/V positions. a, d: anterior, dorsal; as, is, mds: antennal, intercalary and mandibular *en* stripe, respectively; cl: *en* expression in the clypeolabrum; dh: *en* expression in the dorsal hemispheres; hs: *en* head spot; AN, IC, MD, MX: antennal, intercalary, mandibular and maxillary segment, respectively; CL: clypeolabrum; FG: foregut; ML: ventral midline; OA: Bolwig organ/optic lobe anlagen.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Td5
Ppd2
Dd3
Td6
Td4
Ppd9
Ppd5
Dd4
Ppd3
Td8
Ppd6
Dd8
Ppd4
Ppd8
Dd7
Ppd12
Ppd13
hs
Pcd19
Pcd20
Ppd11
as
Ppd14
Ppd15/16
Pcd21
Dd13
Ppd18
Ppd20
a
d
lst11
H
CL
Pad14
Pad13
Pad10
Pcd8
Pad11
Pad8
Pad16
Pad9
Pad18
Pad17
Pad12
Pad5
Pad6
Pcd14
Pcd9
Pcd4
Pav1
Pcv7
Pad7
Pad3
Pcd10
Pcd5
Pad15
Pcv9
Pcv6
Pcv4
Pad4
Pad1
Pcd16
Pcd11
Pcd6
Pcv8
Pcv5
Pcv2
Pcd18
Pcd2
Ppv1
Pcv3
Pcd19
Pcd13
Pcv1
Pcd1
Ppv3
Ppv2
Dv5
FG
Pcd12
Ppd6
Ppd1
Dv6
Dv3
Dv1
Pcd20
Ppd7
Ppd3
Dd2
Dv7
Dv4
Pcd21
Ppd12
Ppd4
Ppd5
Dd6
Dv8
Dv2
Ppd10
Ppd8
Dd3
Dd1
Tv2
Ppd17
Ppd13
Dd8
Dd5
Td1
Td2
Tv3
Ppd14
Ppd11
Dd7
Dd4
Ppd18
Ppd15
hs
Dd10
Td8
Td6
Td4
Td5
Td3
Tv5
is
Ppd20
Ppd16
as

Fig. 3. Identification of individual brain NBs at different embryonic stages. (A) Deadpan (Dpn) expression in the procephalic neuroectoderm by mid stage 8 (mst8) at two different focal planes (flat preparation; anterior is towards top, dorsal is leftwards). Dpn protein is found in two small spots in the ocular (arrowheads in A), and in a stripe-like domain in the antennal ectoderm (black arrows) as well as in the first brain NBs (Pcv7, 9), in a focal plane about 10 μm deeper (A′). Red arrows indicate same positions in the two optic foci, revealing that Pcv7 derives from a small cluster of Dpn-positive ectodermal cells. (B-H) *svp-lacZ* (brown)/Engrailed (blue) double stainings in flat preparations (B,D,F,H; ventral views, anterior (a) is towards the top and dorsal (d) is leftwards) and wholemounts (C,E,G; lateral views, anterior is towards the top and dorsal is towards the left). Individual NBs are identifiable by marker expression, morphology (size), and position relative to each other. Compare with semi-schematic presentation in Fig. 2. (B) Early stage 9 (est9); *svp* is weakly expressed in a subset of NBs. Ectodermal progenitor cells immediately prior to NB formation are marked by brackets (untypically, in this embryo Dv4 develops before Dd7). (C-H) For comparison, whole-mount and flat preparation of the same individuals are shown. (C,D) Late stage 9 (lst9). Note the higher optic resolution and easier identification of NBs in the flat preparation. (E,F) Mid stage 10 (mst10; F, the most dorsal Svp-positive Pcd18 was removed accidentally during preparation). (G,H) Late stage 11 (lst11). a, d: anterior, dorsal; as, is: antennal and intercalary *en* stripe, respectively; cl: *en* expression in the clypeolabrum; hs: *en* head spot; CL: clypeolabrum; FG: foregut.

The procephalic neuroectoderm also forms the anlagen of the adult optic lobes. These precursors are clearly distinguishable from NBs, as their mode of formation is different. They invaginate as separate epithelial primordia from the dorsoposterior ectoderm that subsequently attach to the brain (Green et al., 1993). By stage 12, when the optic lobe primordia start to invaginate, all identified brain NBs have already formed. Some of them are located adjacent to the anterior lip of the optic lobe anlagen, but none is observed to be part of it (data not shown). The optic lobe anlagen will not be considered further in this study.

Glial and sensory precursors

To map the positions of putative glial precursor cells, we investigated the expression pattern of the two glia specific genes, *reversed polarity* (*repo*) (Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994) (using an anti-Repo antibody) and *glial cells missing* (*gcm*) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) (using *gcm* RNA probes; Fig. 4A-E). Like in the ventral nerve cord these two genes are co-expressed in cells of the early brain, with Repo expression starting slightly later than *gcm* at late stage 10 (Fig. 4C). Until late stage 11 more than 20 cells express Repo, most of them being part of the proto- and tritocerebrum (Fig. 4B). Owing to their small size, many of them may represent progeny cells of closely associated NBs (Fig. 4A-C,E). We were able link Repo expression to identified precursor cells in only two cases. In the tritocerebrum we detect Repo in Td7 (Fig. 4A,B,D,E). Because of its position (immediate posterior to the ‘en as’; data not shown) and onset of Repo expression, Td7 possibly represents the serial homologue of the truncal longitudinal glioblast (Halter et al., 1995). A further tritocerebral Repo-positive cell derives from the Repo-negative Td4, as it co-expresses the marker gene *ladybird early* (Fig. 4D). In the tritocerebrum *ladybird early* is expressed in Td4 and its progeny (Urbach and Technau, 2003b). As co-expression of Repo only occurs in part of the Td4 progeny, Td4 appears to act as a neuroglioblast, generating glia and neurones. The identification of all other glia-producing precursors in the brain will require the application of cell lineage tracers.

To find out whether putative sensory organ precursors are included in our NB map, we investigated the expression of *atonal* (*ato*) (Jarman et al., 1993), and the *lacZ* pattern in the enhancer trap strain A37 (Ghysen and O’Kane, 1989), which are indicative of sensory precursor cells (Fig. 4E-G; Fig. 7). Ato expression is less dynamic and appears to be complementary to the genes of the AS-C (see below). From stage 8 to 10, it is found in four small patches in the antennal and preantennal ectoderm (Fig. 7A,C,E). Until stage 11 one protocerebral (ocular Ppd19) and six antennal NBs (Dd9, Dd11, Dd12, Dd13, Dv1, Dv3) derive from these patches. Except for Dd13, no Ato-positive NB co-expresses any gene of the AS-C (Fig. 7D,F,H). In Ato/A37 double staining, co-expression was detected in five stem cells by stage 11 (Dd9, Dd11, Dd12, Dd13 and Dv1; co-expression in Dv3 only until stage 10, later only A37; Fig. 4E-G). Considering their characteristic position within the ectoderm of the antennal primordium (Fig. 4F,G; which is also confirmed by Ato/En double staining; data not shown), it is likely that Dd9, Dd11, Dd12 and Dd13 represent precursors of the larval antennal dorsal organ (Campos-Ortega and Hartenstein, 1997; Schmidt-Ott et al., 1994). Being located in the ventralmost position at the level of the *en* antennal stripe (Fig. 2G, Fig. 4F,G), Dv1 (and perhaps Dv3) possibly represents the precursor of the hypopharyngeal/latero-hypopharyngeal organ (Campos-Ortega and Hartenstein, 1997; Schmidt-Ott et al., 1994). Ato protein, but not A37, is found in the anteriormost protocerebral NB Ppd19 (Fig. 4E, Fig. 7D,F,H), which appears to generate three Ato-positive progeny cells until stage 13, mapping in the dorsolateral protocerebrum (data not shown). Determining how far the other Ato/A37-expressing precursors also contribute cells to the CNS awaits cell lineage tracings.

Taken together, expression of molecular markers like Repo and Ato indicates that glial and some sensory precursors are included in our NB map.

Brain neuroblasts originate from ectodermal domains with distinct mitotic behaviours

Foe (Foe, 1989) subdivided the procephalic ectoderm into several mitotic domains which are characterized as discrete groups of cells synchronously entering the 14th mitotic cycle. As these domains were suggested to represent units of morphogenetic function (Foe, 1989), we attempted to link populations of identified brain NBs to specific mitotic domains. Because time of entry into mitosis varies considerably between mitotic domains, each domain is only recognizable during its period of mitosis but not before or thereafter. Furthermore, almost all procephalic mitotic domains have already completed the 14th mitotic cycle (by early/mid-stage 8) before they give rise to NBs. Therefore, assigning NBs to particular mitotic domains is a demanding task. To trace the arrangement of procephalic mitotic domains during early neurogenesis and the populations of NBs they give rise to, we used a 4D microscope system (Schnabel et al., 1997), which permits continuous

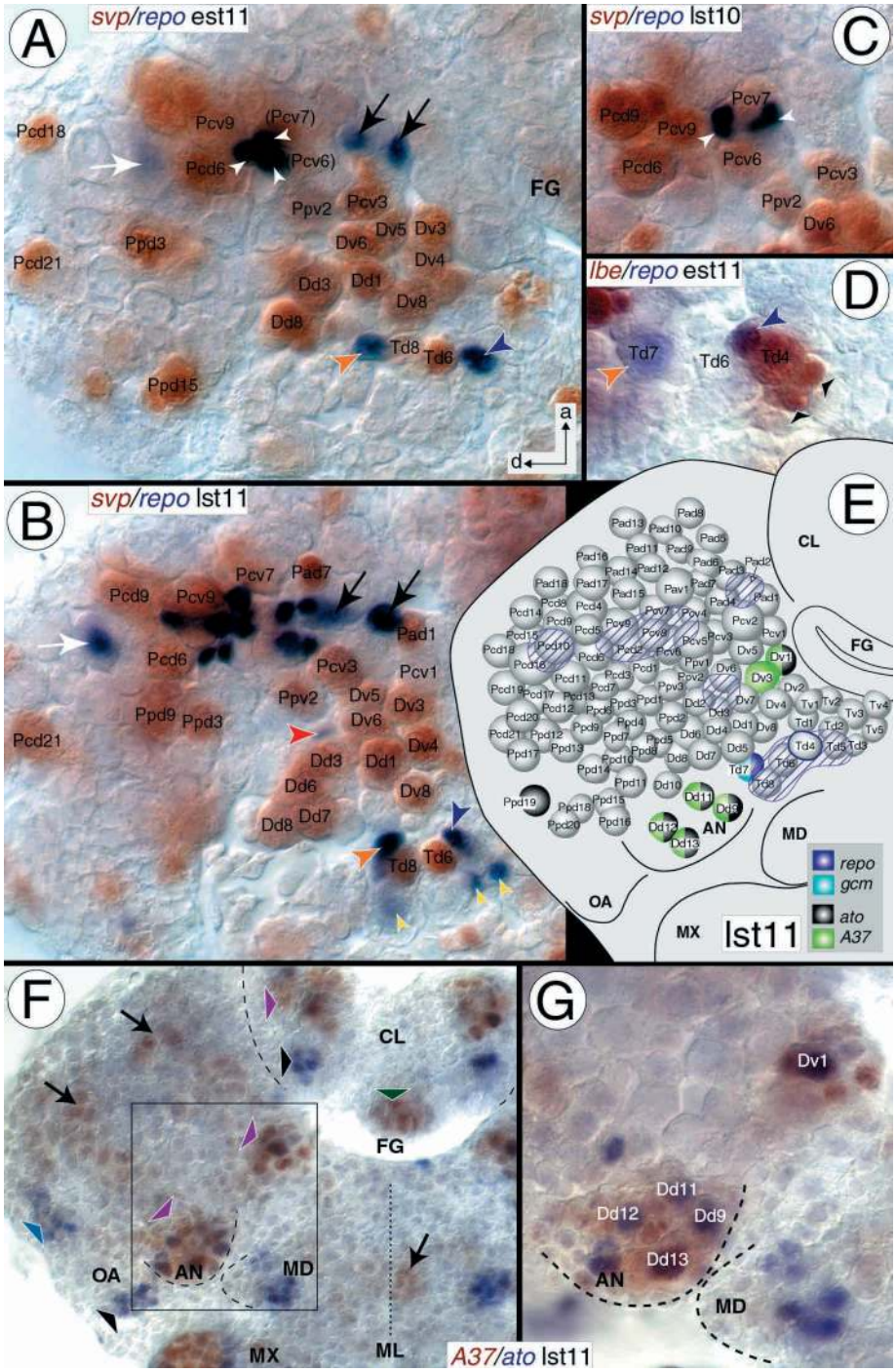

Fig. 4. Expression of markers for glial cells and sensory precursors. (A-C) *svp-lacZ* (brown)/Repo (blue) double stainings between late stage 10 and late stage 11 (as indicated). Identified Svp-positive NBs are designated. By late stage 10, a cluster of two or three small protocerebral cells becomes Repo positive (white arrowheads in A,C; slightly different stages and focal planes). These are the first cells expressing Repo in the CNS (slightly before the longitudinal glioblast in the ventral nerve cord) (Halter et al., 1995), and might belong to the glia cluster 'VPSG' described previously (Hartenstein et al., 1998). Considering their relatively small size, they could represent progeny cells of closely associated early NBs (e.g. Pcv6, Pcv7 or Pcv 9, which might act as neuroglioblasts as they are Repo negative). Slightly later, Repo is found in three further proto- and two tritocerebral cells (A). Two of the protocerebral cells (black arrows) are located ventrally in close vicinity to Pad2 (E, not in focus in A), and one more dorsally (white arrow) (possibly belonging to 'VPSG' and 'DPSG', respectively) (Hartenstein et al., 1998). By late stage 11, about eight additional Repo-positive cells can be detected in the protocerebrum at various positions along the DV axis (B). Considering their small size and distribution, they could be progeny cells of at least two central protocerebral NBs. Furthermore, at this stage three further Repo-expressing cells appear in the dorsal tritocerebrum at the border between the intercalary and mandibular segment (close to Td6, Td8 and Dd9; yellow arrowheads in B). The red arrowhead indicates the first deutocerebral cell expressing Repo. In two cases, glial precursors could be identified: Td7 (orange arrowhead in A,B,D) and Td4. (D) Td4 is a neuroglioblast; Ladybird early (*lbe*)/Repo double staining revealed a glial component (blue arrowhead) of the Lbe-positive Td4 lineage; black arrowheads mark other Lbe-positive daughters of Td4. (E) Because in all other cases it is not possible to link the Repo-labelled cells to identified precursors, their distribution relative to the NB pattern is marked by blue hatching in the semi-schematic presentation. (F,G) Atonal (blue)/A37-*lacZ* (brown) double staining at late stage 11. (F) In the pregnathal head A37-*lacZ* is found at strong levels in the ectoderm of the antennal appendage (AN), in a ventral ectodermal cell cluster near the foregut anlage (FG), and in ectodermal spots in the labral appendage (LR; violet and green arrowheads). Note that A37-*lacZ* is also detected at significantly lower levels in other parts of the procephalic and truncal neuroectoderm (black arrows), which is believed to be not indicative for sensory cells. Ato is co-expressed in subsets of strongly A37-*lacZ* positive cells (violet arrowheads); moreover, Ato is found in a dorsal cell cluster (blue arrowhead), including NB Ppd19 (E), in the primordial Bolwig organ cells of the optic anlage (OA), and in the labral appendage (black arrowheads). Dashed lines contour the outline of the mandibular, antennal and labral appendages; the dotted line contours the outline of the ventral midline (ML). (G) Close-up of the region framed in F at the level of NBs. Indicated are five A37-*lacZ*/Ato co-expressing antennal NBs; considering their position at the basis of the antennal appendage, Dd9, Dd11, Dd12, Dd13 are putative precursors of the Dorsal organ, the ventral Dv1 (and Dv3, which is not in focus) of the hypopharyngeal/latero-hypopharyngeal organ. Expression of *A37-lacZ* and Ato in stem cells is summarized in E. a, d: anterior, dorsal; AN, MD, MX: antennal, mandibular and maxillary segment, respectively; CL: clypeolabrum; FG: foregut; ML: ventral midline; OA: Bolwig organ/optic lobe anlagen.

Table 1. Assignment of subpopulations of brain NBs to mitotic domains

NB population		Mitotic domain
P	Anterior	1
P	Central	B
P	Posterior (dorsal)	5†
P	Posterior (ventral)	9
P	Posterior (dorsalmost)	20?*
D	Main fraction	9
T	Main fraction	2?†

*Dorsal pouch epithelium, Bolwig organ and optic lobe derive from domain 20 (Namba and Minden, 1999).

†Fate-mapping data indicate that dorsoposterior protocerebral (P) and tritocerebral (T) progenitors originate from neuroectodermal regions corresponding to mitotic domains 5 and 2, respectively (Technau and Campos-Ortega, 1985; Schmidt-Ott and Technau, 1994).

D, deutocerebral NBs.

following of cell positions, cell divisions and cell fates in the living embryo (see Materials and Methods). During stages 6-11, the relative positions of ectodermal regions corresponding to particular mitotic domains do not change in principal (Fig. 5C). Brain NBs derive from essentially four or five mitotic domains: domain 1, 5, 9 and B [and possibly domain 2; nomenclature of mitotic domains according to Foe (Foe, 1989)]. We provide a correlation between these domains and subpopulations of brain NBs as summarized in Fig. 5 and Table 1.

The centrally located domain B consists of 50-60 neuroectodermal cells. These cells show no mitotic activity in the peripheral ectoderm (Foe, 1989) (see below). By stage 7/early stage 8, the ectodermal cell layer in this region appears thicker and most of the cells are characterized by an apically narrowed and basally enlarged shape. By middle stage 8, first NBs emerge from this domain (Fig. 5A, Fig. 8A-C). We find that all NBs (about 25) arising from domain B contribute to central parts of the protocerebrum (Fig. 5B). The posteroventrally adjoining domain 9 encompasses about 50 neuroectodermal cells, giving rise to about 10 posterior protocerebral NBs, most (if not all) deutocerebral and presumably some anterior tritocerebral NBs (Fig. 5B,C). Domain 1 covers the anterior part of the procephalon, adjoining domain B anteriorly (Foe, 1989) (Fig. 5A,C). Consistent with its position in the procephalic neuroectoderm and relative to domain B, we find that the anterior population of (about 15) protocerebral NBs originates from domain 1 (Fig. 5B). Domain 5 covers the dorsal part of the procephalon anterior to the cephalic furrow, abutting domains 2 and 9 ventrally, and domains B and 20 anteriorly (Foe, 1989) (Fig. 5A,C). A dorsoposterior subset of about 15 protocerebral NBs develops from domain 5 (Fig. 5B). As neurogenesis in the intercalary segment is strongly delayed (starting at about stage 10; Figs 2, 3), it is hard to ascertain from which mitotic domain tritocerebral NBs arise. However, because during stages 8-11 the relative positions of the mitotic domains do not change, and of NBs to each other and to the outer ectoderm appear to be maintained, it is likely that the posterior portion of tritocerebral NBs originates from the ectoderm posteroventral to domain 9. Thus, we conclude that part (or all) of the tritocerebral NBs develop from domain 2 (Fig. 5A-C).

Different modes of neuroblast formation in the procephalic neuroectoderm

Next, we asked whether the differences between the mitotic domains, like onset of mitosis and orientation of the mitotic spindle, might have an impact on the process of NB formation in the procephalic neuroectoderm. To clarify this we focused on the procephalic mitotic domains B, 1, 5 and 9, the mitotic behaviour of which is distinct (Foe, 1989), and from which most of the brain NBs derive.

Four-dimensional microscopy data show that cells in mitotic domain B, as opposed to all other procephalic mitotic domains, do not divide prior to NB delamination (as confirmed by anti α-Tubulin antibody staining; Fig. 8A-C) supporting earlier observations (Foe, 1989). By stage 7/8 neuroectodermal cells in domain B gradually enlarge on the basal end and delaminate successively as NBs, thereby losing their slender contact to the apical ectodermal surface (Fig. 6A, Fig. 8A-C).

By early stage 8, cells in domain 9 move within the neuroectodermal layer in the apical direction and round up. Subsequently, these cells become reintegrated into the ectoderm (Fig. 6B). Almost all domain 9 cells undergo this apical movement that for each cell lasts about 2 minutes (Fig. 6D; and for complete domain 9 about 15-18 minutes). This is much faster than the process of basally directed delamination of NBs in the procephalic neuroectoderm (normally more than 10 minutes). The spatiotemporal pattern of apical movement of domain 9 cells appears to be roughly reproducible, initiated by four or five neuroectodermal cells at the edge (close to domain B) and then spreading centrifugally; consequently, immediately adjacent cells undergo this movement. However, as the number of cells moving apically is larger than the number of later arising NBs, it seems that not all domain 9 cells are NB progenitors. We find that apical movement of cells can be achieved in two different ways: delamination and directed mitosis. In the first case (Fig. 6B, part a, Fig. 6D) apical movements precede the basally directed delamination of NBs. Cells show the morphological signs of delamination, e.g. the cytocortical bundles of microtubules appear to rearrange and the cell becomes bottle-shaped with a transient narrow neck directed basally. Upon reintegration into the ectoderm some of these cells move basally to finally delaminate as NBs (Fig. 6B, part a_1). Others stay within the peripheral neuroectoderm and presumably become epidermoblasts (Fig. 6B, part a_2). In the second case (Fig. 6B, part b) the ectodermal cell divides with its mitotic spindle oriented perpendicular to the ectodermal surface (Foe, 1989). Thus, one daughter cell becomes located apically, reintegrates into the ectoderm and presumably develops as an epidermoblast. The sibling cell is deposited in the basal direction to become a NB.

In mitotic domains 1 and 5 all cells undergo a division in parallel to the ectodermal surface (Foe, 1989) before first NBs delaminate from these domains. Most of these divisions appear to result in one daughter cell which subsequently delaminates from the ectoderm as a protocerebral NB, and a second precursor which remains within the outer ectoderm and presumably acts as an epidermoblast (Fig. 5C).

Taken together, we find different modes according to which brain NBs arise from the neuroectoderm, and which are correlated with distinct mitotic domains. Whereas the modes of NB formation we find in mitotic domain B (Fig. 6A) and

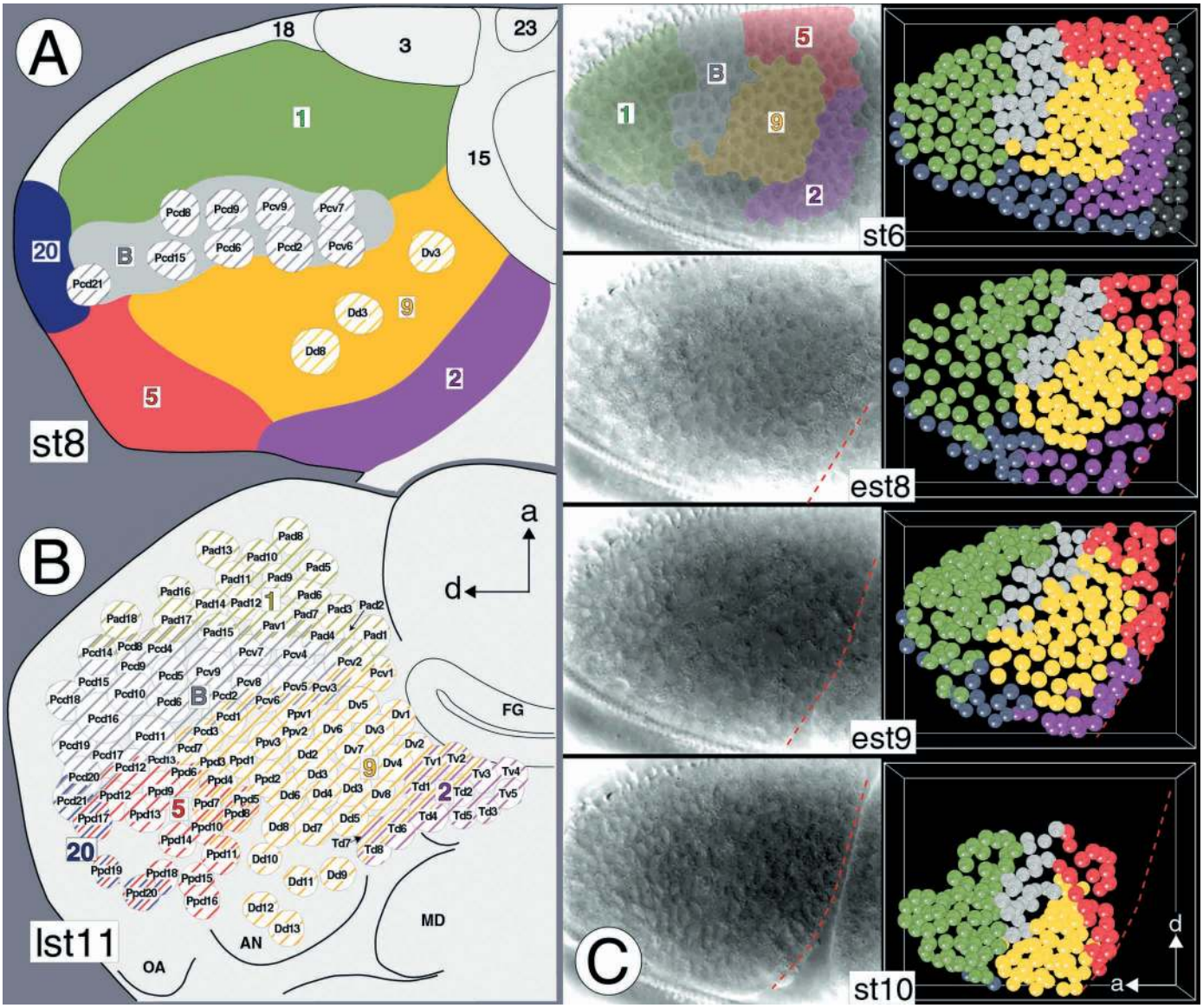

Fig. 5. Procephalic mitotic domains and the origin of brain NBs. (A) Projection of the spatial arrangement of mitotic domains (Foe, 1989) onto a schematic flat preparation at stage 8 [anterior (a) is towards the top, dorsal (d) is towards the left] based on 4D microscopic data (shown in C) as well as anti-alpha-Tubulin antibody staining (see Fig. 8). Different colours mark the mitotic domains 1, 2, 5, 9, 20 and B. The earliest set of NBs derives from mitotic domains B and 9. (B) Schematic flat preparation at late stage 11. Mitotic domains 1, 2, 5, 9 and B (and perhaps 20) contribute NBs to the embryonic brain (see also Table 1). Coloured hatched lines mark subpopulations of NBs that derive from the respective domains (compare A). (C) 4D microscopic analysis. Left panel depicts Nomarski pictures taken from an in vivo timelapse sequence of the same embryo between stages 6 and 10 (st6, stage 6; est8, early stage 8; est9, early stage 9; st10, stage 10; lateral views of the procephalic ectoderm in the same focal plane; anterior is towards the left, dorsal is towards the top; red, stippled lines mark the cephalic furrow). Right panel demonstrates computer models of the pictures on the left side (see Materials and Methods); each ectodermal cell is represented by a dot, the centre of which corresponds to the centre of the nucleus. According to their typical mitotic behaviour (e.g. time point of mitosis; orientation of mitotic spindle), ectodermal cells can be grouped into distinct mitotic domains (Foe, 1989). Colour code indicates cells belonging to the same mitotic domain. The movements and fate of each blastodermal cell can be traced through ongoing development (here shown until stage 10). Posterior black dots at stage 6 indicate cells that subsequently move into the cephalic furrow and thus out of the focal planes (not shown in following stages). Ventral dark grey dots indicate cells belonging to other mitotic domains that were not traced because they move out of focal planes. Note the slight rotation of the head ectoderm: dorsal cells move towards posterior positions and posterior cells towards more ventral positions. Importantly, despite these movements and the delamination of NBs, the relative positions among mitotic domains and among cells within each domain do not change in principal. a, d: anterior, dorsal; AN, MD: antennal and mandibular segment, respectively; FG: foregut; OA: Bolwig organ/optic lobe anlagen.

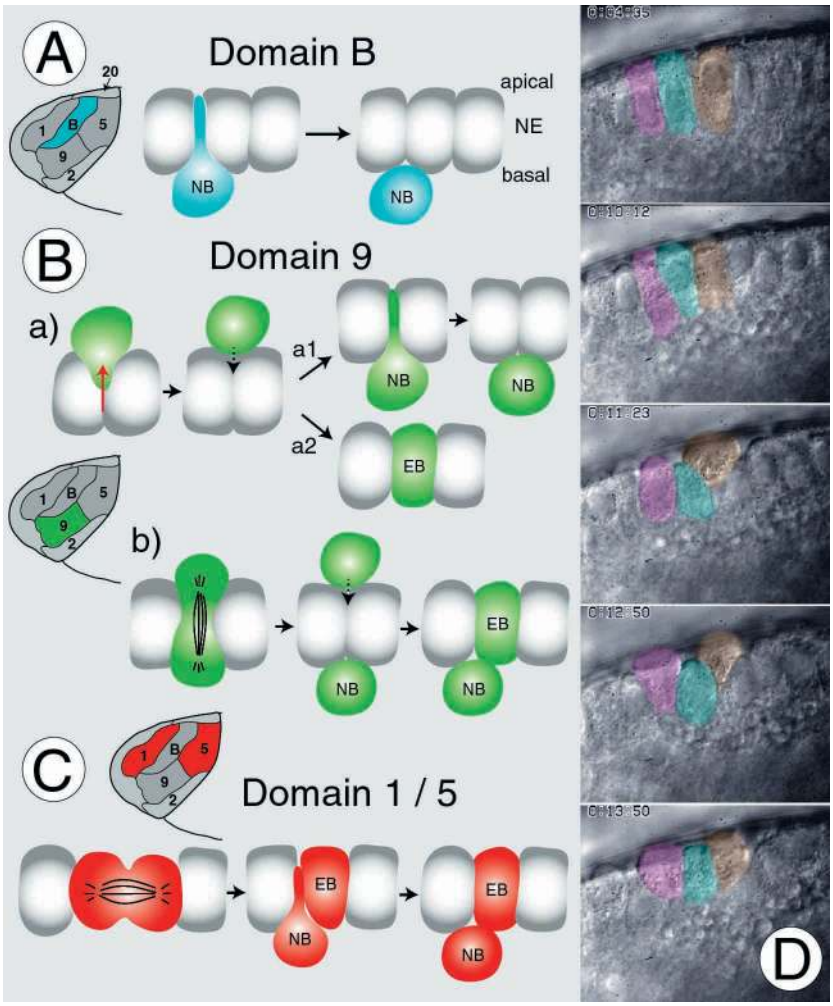

Fig. 6. Modes of brain NB formation differ between mitotic domains. (A) In mitotic domain B, NBs form by basally orientated delamination from the neuroectoderm (NE). The scheme on the left highlights, in a lateral view, the arrangement of mitotic domains (at stage 7) that contribute to the embryonic brain. (B) NE cells in domain 9 move apically (red arrow in Ba; see also D) and subsequentially reintegrate into the NE layer to delaminate as NBs (Ba1) or remain in the ectoderm to develop as epidermoblasts (EB in Ba2). Other cells in domain 9 divide perpendicular to the ectodermal surface (as indicated by the mitotic spindle; Bb); one daughter cell moves apically but later reintegrates into the NE as an epidermoblast, the other is deposited basally to become a NB. (C) In domains 1 and 5, NE cells divide parallel to the ectodermal surface; thereafter one of the two daughter cells usually stays in the peripheral ectoderm as an epidermoblast, the second delaminates as a NB. (D) Timelapse sequence focusing on part of the NE layer in mitotic domain 9 a few minutes after cephalic furrow formation. Sequence of pictures shows the apically directed movement of NE cells. First, the yellow NE cell leaves and reintegrates into the layer, followed by the red cell; each cell needs about 2 minutes to achieve this movement (time is depicted in pictures). Note the changing shape of apically moving cells (compare with Ba).

1/5 (Fig. 6C) correspond to the behaviour of cells in the truncal neuroectoderm (Bossing et al., 1996; Hartenstein and Campos-Ortega, 1984), those in domain 9 appear to be brain specific.

The pattern of proneural gene expression in the procephalic ectoderm and brain neuroblasts

Considering the differences in the patterns and modes of NB formation between the developing ventral nerve cord and the brain, and the fact that NB formation is promoted by proneural genes, we investigated in detail the expression of members of the Achaete-Scute-Complex (AS-C) (for a review, see Campos-Ortega, 1995) *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) during early brain development (stages 8-11). In double labelling with *engrailed* expression as a segmental marker, we precisely determined the relative position of proneural gene expression domains within the procephalic neuroectoderm, as well as the expression in the descending NBs (summarized in Fig. 7). The rapidly changing pattern of L'sc expression roughly foreshadows the spatiotemporal development of brain NBs [Fig. 7B,D,F,H; for description of l'sc expression see also Younossi-Hartenstein et al. (Younossi-Hartenstein et al., 1996)]. About 60% of all NBs formed until stage 11 express L'sc, including almost all NBs formed during stages 8 and 9. The pattern of Ac expression during stage 8 and 9 is largely complementary to L'sc (Fig. 7A-D). *sc* is not expressed before stage 10 (Fig. 7E-H).

Co-expression of proneural genes in brain NBs appears to be rare and transient; e.g. by stage 8, four out of 16 NBs show co-expression of two proneural genes (*ac* and *l'sc*), by stage 9 co-expression occurs in only one out of 27 NBs (Fig. 7B,D,F,H). Despite the general correspondence between the pattern of proneural gene expression in the neuroectoderm and deriving NBs, some NBs express proneural genes at detectable level only after their formation, i.e. upon delamination from the neuroectoderm, which at that time does not express the respective gene (e.g. *ac* in Dd3, Dv6, Pad4, Pcd15, Pcd16 and Pcv3, or *l'sc* in Pcd17, Pcd21; Fig. 7B-H). Likewise, in the trunk *l'sc* expression was found in NB3-5, but not in the corresponding proneural cluster (Skeath et al., 1994). However, a subset of brain NBs (about 25%) does not express any of the investigated proneural genes at a detectable level. This is mostly observed in late developing NBs (e.g. for five stage 10 NBs and about 22 stage 11 NBs; Fig. 7F,H), implying that other proneural genes might exist.

Proneural gene expression in the procephalic neuroectoderm is found in patches of significantly varying size. *ac*, *sc*, *l'sc* and *ato* are all expressed in small proneural clusters (of five to seven cells) as well as in larger ectodermal domains. The dynamics of gene expression in the small clusters reflects the process of singling out of the presumptive NBs, i.e. expression initially occurs in all cells of a cluster, but after segregation it is only maintained in the respective NB. Proneural gene expression in larger ectodermal domains appears to be regulated differently. For example, the large *l'sc* domain which during stages 7-10 spans most of the procephalic neuroectoderm, gives rise to more than one NB (Fig. 7) (Younossi-Hartenstein et al., 1996). Accordingly, *l'sc*

expression within this 'proneural cluster' (equivalence group of cells with NB-forming potential) shows a distinct dynamic: although NBs after segregation express L'sc at high levels, all surrounding cells do not lose it; thus, presumably retaining their potential to become a NB. For further details of proneural gene expression see Fig. 7.

Brain neuroblasts can develop from adjacent neuroectodermal cells

In the ventral neurogenic ectoderm of the trunk, each proneural cluster of five to seven cells gives rise to a single NB. A lateral inhibition process mediated by the neurogenic genes prohibits more than one cell from each cluster adopting a neural fate (for a review, see Artavanis-Tsakonas et al., 1991; Campos-Ortega, 1993). Thus, in the truncal neuroectoderm, immediately neighbouring cells are very unlikely to develop as NBs. The fact that, in the head, expression of proneural genes is found in larger domains of the neuroectoderm and in groups of NBs corresponding to these domains (see above), raises the possibility that in the procephalic neuroectoderm adjacent cells may develop as NBs. To test this hypothesis, we traced the segregation of individual NBs from the procephalic ectoderm more closely. First, we performed double labelling with antibodies against α-Tubulin and Dpn (Fig. 8A-C). In domain B, most of the developing NBs transiently show a thin, apically directed process, which is visible until the NB has completely delaminated (Fig. 6A). In some cases, we observe that, consistent with the subectodermal position of the delaminating NBs, their corresponding apical processes are also in immediate vicinity of each other (e.g. Pcd2, Pcd4 and Pcv9 in Fig. 8B,C), suggesting that these NBs derive from neighbouring neuroectodermal cells. To obtain more direct evidence for this spatial relationship, we applied 4D microscopic analysis (see Materials and Methods). This allowed us, in vivo, to trace back the origin of a subset of identified NBs to their corresponding neuroectodermal progenitors in the blastoderm (stage 6;

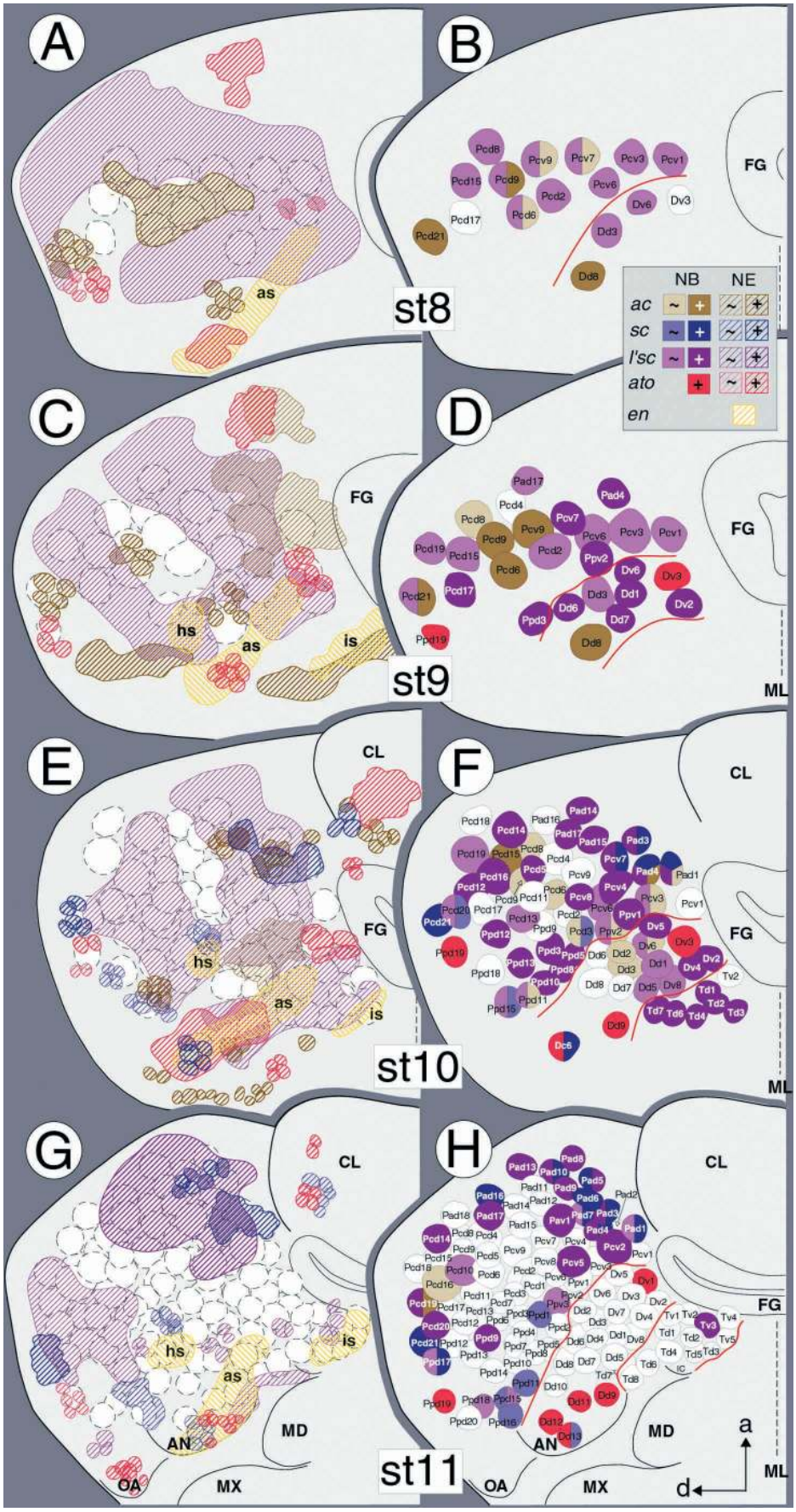

Fig. 7. Expression of proneural genes in the procephalic neuroectoderm and brain NBs. Semi-schematic presentation of the expression patterns of *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *atonal* (*ato*) (from stage 8 to 11) at the level of the procephalic ectoderm (A,C,E,G; positions of underlying NBs are marked by broken lines) and at the level of individual brain NBs (B,D,F,H) (see key for neuroblasts, NB and neuroectoderm, NE; high (+) and low (~) levels of expression are indicated by colour intensities). In the peripheral ectoderm (A,C,E,G) single cells are outlined when expression domains comprise less than eight cells. Additionally, the expression of *engrailed* (*en*) in the peripheral ectoderm is highlighted. Orientation as in Fig. 2. By stage 7/8, L'sc protein is detected in a large domain covering the central neuroectoderm (encompassing mitotic domain B and 9) from where first proto- and deutocerebral NBs develop (A,B). By stage 10, L'sc expression has expanded into other regions of the procephalic neuroectoderm (E), but by late stage 11 becomes confined to several smaller ectodermal domains, and especially to the dorsomedial neuroectoderm from where anteriormost protocerebral NBs emerge (G,H). Of all investigated proneural genes only L'sc is found in the intercalary NE (E,G) and about seven deriving tritocerebral NBs (F,H). Ac expression is detected by stage 8 in a small dorsal ocular and antennal group of neuroectodermal cells (from which Pcd21 and Dd8 derive). Furthermore, Ac is expressed in a large domain of the central procephalic neuroectoderm where four or five NBs, which co-express L'sc derive from (A,B). During stage 9 Ac is found in several large domains at different sites of the procephalic neuroectoderm (C), from which, by stage 10, about nine additional, weakly Ac-positive NBs emerge (D,F). During stage 10, Ac expression decreases in the peripheral ectoderm and is, by stage 11, confined to two most dorsal NBs (Pcd16 and Pcd19; E-H). *sc* is not expressed before stage 10, when it is found in about six small patches in the antennal and preantennal ectoderm, which (except for a patch in the clypeolabral ectoderm) give rise to single, or small groups of, NBs. Furthermore, it is strongly expressed in a domain of the anteriormost procephalic neuroectoderm and a corresponding group of about seven protocerebral NBs (E-H). Ato protein is expressed in a cluster of about six ocular ectodermal cells (A) that presumably represent the 'proneural cluster' from which Ppd19 derives (D). Dv3 expresses Ato only after formation (compare B with D) in contrast to the adjacent Ato-positive Dv1 (H), which develops from the Ato-expressing proneural cluster in the ventral antennal ectoderm (C,E). By stage 11 Ato is additionally expressed in primordial cells of the optic anlagen (OA in G). a, d: anterior, dorsal; as, is: antennal and intercalary *en* stripe, respectively; hs: *en* head spot; AN, MD, MX: antennal, mandibular and maxillary segment, respectively; CL: clypeolabrum; FG: foregut; ML: ventral midline; OA: Bolwig organ/optic lobe anlagen.

Fig. 8D-G). We focused on early NBs (stage 8; Fig. 2) most of which derive from domain B (Fig. 5A). As NBs from domain B do not divide before delamination from the ectoderm (see Fig. 6A) they are rather large, facilitating their identification in vivo. We traced the origin of a group of about 10 identified late stage 8 NBs (Fig. 8C,F,G). We find that the spatial relationships of these cells in the NB layer (Fig. 8D,E) closely correspond to their previous arrangement in the neuroectoderm, where they represent a group of adjacent cells (Fig. 8F,G). Also in domains 1, 5 and 9, we found cases in which two or more neighbouring cells develop as NBs (data not shown). We conclude, that in contrast to the situation in the truncal neuroectoderm, adjacent cells in the procephalic neuroectoderm (belonging to the same 'proneural cluster') can adopt neural fate. Thus, the process of lateral inhibition appears to be less efficient in the procephalic neuroectoderm, allowing more cells to follow their primary neural fate. In domain B, where many adjacent cells develop as NBs, lateral inhibition may even be entirely lacking.

DISCUSSION

The spatiotemporal pattern of brain neuroblast formation is stereotypical

In earlier studies, the development of the procephalic NB pattern has been partially described. Using morphological criteria, Hartenstein and Campos-Ortega (Hartenstein and Campos-Ortega, 1984) found about 70 brain NBs, and based on the expression of *l'sc*, *ase* and *svp*, Younossi-Hartenstein et al. (Younossi-Hartenstein et al., 1996) subdivided a total of 75-80 brain NBs per hemisphere into 23 groups of one to five NBs each. In this study, we describe for the first time the development of the procephalic NB pattern at the level of individually identified NBs. Using general NB markers (*dpn*, *ase*) and morphological criteria, we have identified about 105 brain NBs in each hemisphere and documented their temporal sequence of formation as well as their positional relationships between developmental stages 8 and 11. Identities of the individual NBs at different stages are confirmed by the analysis of about 30 other marker genes expressed in single, or subsets of, brain NBs (this paper) (Urbach and Technau, 2003a; Urbach and Technau, 2003b). The differences in NB numbers, when compared with earlier studies, might be due to the fact that, by generating flat preparations (see Materials and Methods) instead of wholemounts (as in previous reports), we gained a significantly higher resolution of the NB layer (compare Fig. 3C,E,G with Fig. 3D,F,H). We find that during formation of the brain anlage, new NBs are continuously added. We did not observe a segregation of brain NBs in waves, which has been suggested to occur in analogy to the trunk (Younossi-Hartenstein et al., 1996). Differences in the spatiotemporal pattern of NB development between trunk and procephalon are not surprising considering the patterns of expression of proneural genes (see below), and the fact that brain NBs derive from distinct mitotic domains (see below), the size and proliferation properties of which (e.g. onset of mitosis, orientation of the mitotic spindle) are heterogeneous and different to the trunk (Foe, 1989). The largest region of the embryonic brain is the protocerebrum, which consists of about 72 NBs. Its extended size, when compared with the deutocerebrum (about 21 NBs) and tritocerebrum (about 13 NBs), is in agreement with the hypothesis that the protocerebrum is composed of two neuromeres (Urbach and Technau; 2003a).

The brain neuroblast map includes glial and sensory progenitor cells

In the trunk, about a quarter of all embryonic NBs generate both neurones and glia (they are appropriately called neuroglioblasts) or only glia (glioblasts) (Bossing et al., 1996; Schmidt et al., 1997). In the brain, a complex pattern of glia is formed (Hartenstein et al., 1998), but their progenitors have so far not been identified. Our data provide first evidence for the existence of a neuroglioblast (Td4) in the embryonic brain. Furthermore, we also have indications for the existence of a

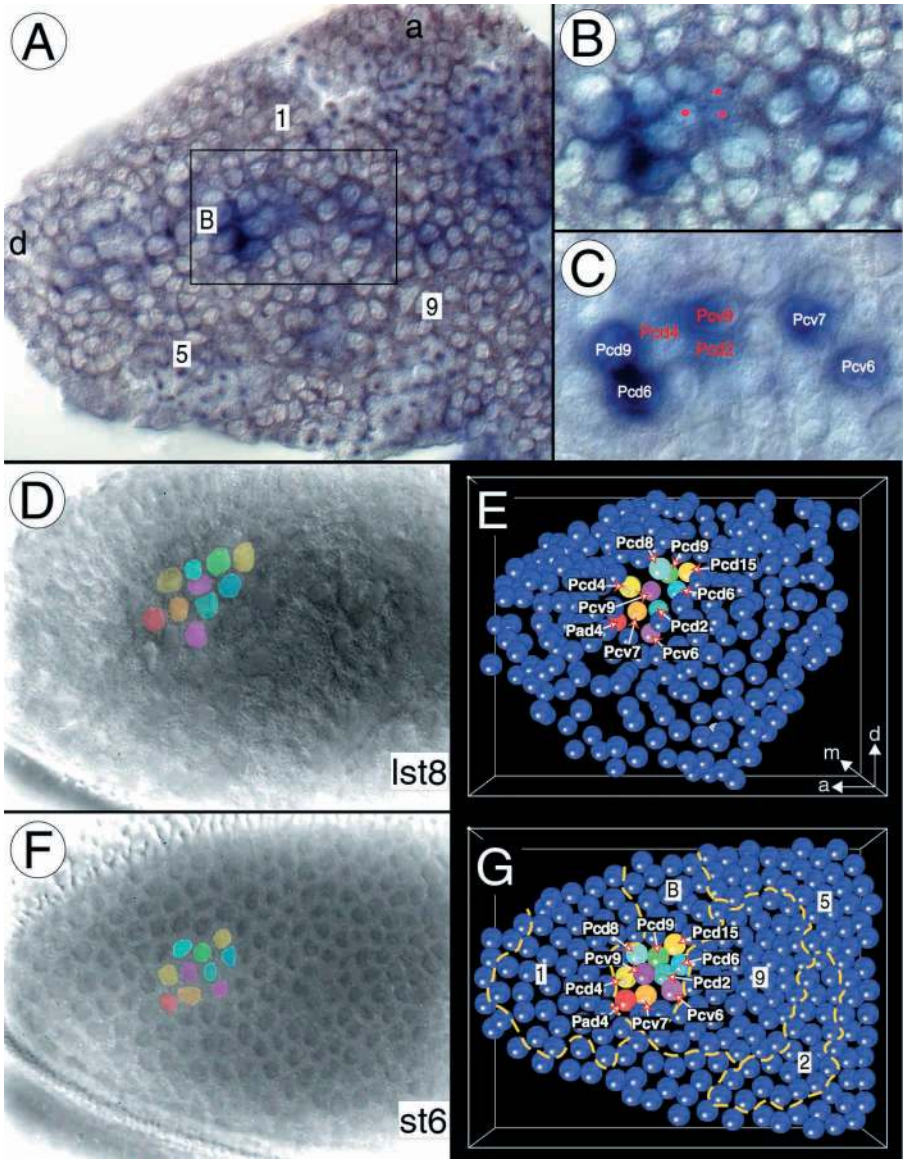

Fig. 8. Brain NBs develop from neighbouring neuroectodermal cells (in mitotic domain B). (A-C) Anti α-Tubulin/anti Dpn double staining of a late stage 8 flat preparation; anterior (a) is towards the top and dorsal (d) towards the left. (A) Anti-α-Tubulin (brown) highlights the outline of ectodermal cells and indicates cells in mitosis (e.g. some posterior cells of domain 5) (Foe, 1989). Position of mitotic domains 1, 5, 9 and B is indicated. Dpn protein (blue) is expressed in ectodermal domains; rectangle encloses most of the ocular Dpn expression that is part of mitotic domain B and gives rise to first brain NBs (compare with Fig. 2A and Fig. 5). At that stage, domain B cells are normally distinguished from domain 1 and 5 cells (most of which have already undergone mitosis) by their larger size. (B,C) Higher magnification of the region framed in A at two different focal planes: the apical level of the peripheral ectoderm (B) and about 20 μm deeper at the level of NBs (C). Red dots in B indicate transient apical cellular processes of three adjacent cells in the centre of domain B. In C, individual Dpn-positive NBs deriving from ocular Dpn domains are assigned. The adjacent apical cellular processes in B belong to the neighbouring NBs marked by red inscription in C. (D-G) 4D microscopic analysis indicates that the population of stage 8 protocerebral NBs derives from adjacent neuroectodermal progenitor cells of mitotic domain B. The origin of identified late stage 8 NBs (D,E) is traced back to the neuroectoderm at stage 6 (st6; F,G) using *z*-stack timelapse recordings (see Materials and Methods). (D,F) In vivo Nomarski pictures (lateral view; anterior is towards the left and dorsal is towards the top) at the level of NBs (D, focal plane at about 25 μm depth) and peripheral ectoderm (F; focal plane at about 8 μm depth). (E,G) Corresponding 3D computer models. Nuclei of identified NBs (as assigned in E) and of their corresponding neuroectodermal precursors (F,G) are indicated by the same colour code. Nuclei of other ectodermal cells are dark blue (E,G). The borders of mitotic domains 1, 2, 5, 9 and B are outlined in G. a, d, m: anterior, dorsal, medial.

glioblast (Td7). The identification of the other glial progenitor cells will require a comprehensive cell lineage analysis. Considering the spatiotemporal pattern of Repo and *gcm* expression, we speculate that – corresponding to the situation in the trunk – most of these progenitors represent neuroglioblasts born at early stages (stage 8/9). Furthermore, in the trito- and deutocerebrum, most glial cells appear to originate from dorsal sides of the neuroectoderm, which express the gene *muscle segment homeobox* [*msh*; for procephalic expression of DV patterning genes see Urbach and Technau (Urbach and Technau, 2003a)], again resembling the situation in the trunk (Isshiki et al., 1997; Schmidt et al., 1997). Whether *msh* is required for proper development of these brain NBs and their glial progeny, as has been shown in the trunk (Isshiki et al., 1997), remains to be settled. However, in contrast to the trito- and deutocerebral brain regions, and the ventral nerve cord, the sites of origin of glial cells in the protocerebrum do not appear to be mainly confined to dorsal positions. This may be due to the profound differences in the expression pattern of DV genes in the preantennal neuroectoderm (Urbach and Technau, 2003a).

Cell lineage tracing in the trunk has indicated that there is a spatial overlap between proneural clusters that give rise to CNS and ventral PNS progenitors (the NB 4-3 and 4-4 lineages each include a sensory subclone) (Schmidt et al., 1997), implying that both types of progenitors can develop in close vicinity. To find out if PNS and CNS precursors intermingle in the procephalon, we applied molecular markers that have been used to label sensory organ precursors (SOPs) in the trunk (Dambly-Chaudiere and Leyns, 1992; Ghysen and O'Kane, 1989; Jarman et al., 1993; Younossi-Hartenstein and Hartenstein, 1997). We identified about six putative SOPs (four

dorsal and two ventral) in the vicinity of CNS precursors. Regarding their position, these can be assigned to the dorsal organ and the hypopharyngeal/latero-hypopharyngeal organ (Campos-Ortega and Hartenstein, 1997). Whether these sensory precursors share common lineages with CNS cells will have to be clarified by lineage analysis. We identified further putative SOPs in the procephalon (precursors of the labral sensory organs and the Bolwig organ) (Campos-Ortega and Hartenstein, 1997), which we did not include in the NB map as they are clearly separated from CNS precursors.

The relationship between embryonic and postembryonic neuroblasts

In the embryonic CNS, NB size decreases with each division, and (except for five brain NBs on either side) NBs cease to proliferate by stage 16 when they are no longer identifiable. After a period of mitotic silence in the late embryo (stage 17) and first instar larva, a population of large postembryonic NBs becomes visible in the peripheral CNS cortex and commences proliferation to produce large numbers of cells of the adult CNS (Prokop and Technau, 1994; Truman and Bate, 1988). For the ventral nerve cord it has been shown that postembryonic NBs originate from embryonic NBs (members of the same lineages) and may even represent identical cells (Prokop and Technau, 1991). About 23 postembryonic NBs have been identified per thoracic hemineuromere in the larva (Truman and Bate, 1988) compared with 31 embryonic NBs (Doe, 1992). Thus, about 75% of the thoracic embryonic NBs resume proliferation in the larva. Interestingly, this coincides with the ratio of NBs found in the embryonic and larval brain. About 80-85 NBs have been described to proliferate in each larval brain hemisphere (Ito and Hotta, 1992). Compared with the number of about 105 embryonic brain NBs found in this study, this suggests that about 78% of the embryonic NBs resume proliferation in the larval brain.

Distinct modes of neuroblast formation are related to mitotic domains

Foe (Foe, 1989) showed that spatiotemporal mitotic patterns arise in the *Drosophila* embryo upon onset of gastrulation (from stage 7), and she defined groups of cells, termed mitotic domains, that enter mitosis (cycle 14) in close synchrony with each other, but out of synchrony with cells of other mitotic domains. She found the borders of the domains to be precisely specified and their arrangement to be conspicuously different in head and trunk. Based on this reproducible pattern and the comparison with fate maps (e.g. Hartenstein and Campos-Ortega, 1985), Foe suggested that the mitotic domains of cycle 14 represented units of morphogenetic function. In order to trace the origin of brain NBs back to the ectoderm and to link them to particular mitotic domains, we used 4D microscopy. As proposed by Foe (Foe, 1989) we found that NBs derived from domains 9 and B. In addition, we observed brain NBs descending from domains 1, 5 and, most probably, 2. However, we cannot exclude the possibility that other mitotic domains (located more ventrally or dorsally) may also participate in the formation of the brain anlage. For example, domain 20, which was recently shown to give rise to the Bolwig organ and optic lobe (Namba and Minden, 1999), may contribute to some of the most dorsal brain NBs (see Fig. 5).

Furthermore, we find that the formation of brain NBs is achieved through several different modes that are related to the mitotic domain of origin. Most domain B cells do not divide in the peripheral ectoderm and delaminate as early NBs, which is analogous to the behaviour of early NBs (S1/S2) in the trunk (Bossing et al., 1996; Schmidt et al., 1997). Neuroectodermal cells in domains 1, 2 and 5 divide in parallel to the ectodermal surface, and usually one of the daughters subsequently delaminates as a NB. Similarly, precursors of late delaminating NBs (S3-S5) in the trunk divide once in the neuroectoderm to generate one neuroblast and one epidermoblast (Schmidt et al., 1997). Domain 9 cells normally divide perpendicular to the ectodermal surface (Foe, 1989) to produce a neuroblast and an epidermoblast. However, we observed that some cells in domain 9 delaminated as NBs without a previous division. This indicates that not all cells within this mitotic domain strictly follow the same mitotic pattern. Although most parts of the brain descend from NBs, recent data have shown that some parts are not formed by typical NBs: small 'placode'-like groups of ectodermal cells close to the head midline invaginate during stage 13 (long after brain NB formation has ceased) and contribute subpopulations of cells to the brain (Dumstrei et al., 1998; Noveen et al., 2000; Younossi-Hartenstein et al., 1996).

Distinct modes of neuronal precursor formation also appear to exist in the developing vertebrate brain. Although neurogenesis in vertebrates generally does not involve delamination of precursors from the neuroectoderm (for a review, see Arendt and Nübler-Jung, 1999), in the zebrafish neuronal progenitors have been observed to delaminate from the neuroepithelium of the inner ear (Haddon and Lewis, 1996). Furthermore, it has been shown for part of the chick neural plate that neighbouring cells can adopt neural or epidermal fate. A functional homologue of the fly proneural genes (*cash4*) is expressed heterogeneously within these cells raising the possibility that, as in *Drosophila*, neural precursors are specified on a cell-by-cell basis through high levels of proneural gene expression (Brown and Storey, 2000).

Expression of AS-C genes differs between head and trunk and does not cover the entire neuroectoderm

In the trunk, genes of the AS-C are expressed in segmentally reiterated, proneural clusters. Their position and size are governed by the combined activity of DV patterning genes and pair-rule genes (Skeath and Carroll, 1992; Skeath et al., 1994). In the procephalic neuroectoderm, the size of 'proneural clusters' is variable. AS-C gene expressing domains are generally much larger than in the trunk [for *l'sc* see also Younossi-Hartenstein et al. (Younossi-Hartenstein et al., 1996)]. We find no indications for a segmental patterning of proneural domains in the procephalon, which is presumably due to the lack of pair-rule gene expression. It has been suggested that, instead of pair-rule genes, head gap genes activate proneural gene expression (e.g. of *l'sc*) (Younossi-Hartenstein et al., 1997). The extended expression of gap genes (e.g. of *otd* and *tll*) (Urbach and Technau, 2003b; Younossi-Hartenstein et al., 1997) would explain the large size of most of the procephalic proneural domains.

Although genes of the AS-C are abundantly expressed and required for NB formation in wild-type trunk and procephalon, a substantial proportion of NBs is still formed in the trunk (Jimenez and Campos-Ortega, 1990) and head (Younossi-

Hartenstein et al., 1997) of embryos that carry a deletion of the entire AS-C. Accordingly, in about 25% of the identified brain NBs, as well as in the corresponding neuroectoderm, we find no expression of genes of the AS-C. Interestingly, the dynamics of expression of a number of further genes is similar to proneural genes (e.g. *ato*, *dpn*; for *eyeless*, *huckebein*, *intermediate neuroblast defective*, *ventral nervous system defective*, *muscle segment homeobox*, *runt*) (see Urbach and Technau, 2003a; Urbach and Technau, 2003b), but so far a proneural function for these genes in the procephalon is not substantiated. In the trunk, a proneural function of *vnd* is suggested because in *vnd* mutants 25% of NBs (comprising a set of NBs that is complementary to that lacking in AS-C mutants) are missing (Jimenez and Campos-Ortega, 1990). Similarly, a loss of a few trunk NBs is observed in *ind* mutants (Weiss et al., 1998). It is speculated that *vnd* and *ind* promote NB formation in the truncal neuroectoderm by proneural-dependent and -independent pathways (Jimenez et al., 1995; McDonald et al., 1998; Skeath et al., 1994; Weiss et al., 1998). Their restricted expression in parts of the procephalic neuroectoderm (Urbach and Technau, 2003a) is compatible with a proneural function of *vnd* and *ind* also in the procephalon. However, for a small number of late developing brain NBs, we find that they and their corresponding neuroectoderm express neither genes of the AS-C nor *vnd* or *ind*. This supports the assumption that in the procephalic neuroectoderm further genes with proneural function might exist.

Reduced efficiency of lateral inhibition among cells of the procephalic neuroectoderm

In the trunk, proneural clusters are defined by proneural gene expression and represent equivalence groups in which all cells have the primary fate to become NBs (e.g. Martin-Bermudo et al., 1991; Skeath and Carroll, 1992). Based on cell-cell interactions, a lateral inhibition process mediated by the neurogenic genes (Notch signalling pathway), progressively restricts proneural gene expression to a single cell, the future NB (for a review, see Campos-Ortega, 1993). In this study, we provide direct evidence that at least in some parts of the procephalic neuroectoderm (e.g. in part of domain B), NBs originate from neighbouring neuroectodermal progenitor cells, which belong to the same 'proneural cluster'. Although the procephalic neuroectoderm also gives rise to epidermal progenitor cells (Technau and Campos-Ortega, 1985) based on the activity of neurogenic genes (as indicated by the hyperplasic brain in neurogenic mutants) (Lehmann et al., 1981), our data suggest that in parts of the procephalic neuroectoderm lateral inhibition is less efficient or even absent. This assumption is further corroborated by experimental data. HRP-injection experiments showed that the ratio between neuronal and epidermal precursors differs significantly between the neuroectoderm of the trunk and head, as a much higher proportion of neuroectodermal cells assumes a NB fate in the procephalon (Schmidt-Ott and Technau, 1994; Technau and Campos-Ortega, 1985). Accordingly, laser ablation of cells in the procephalic neuroectoderm failed to cause defects in the larval epidermis (Jürgens et al., 1986). Cells transplanted from the truncal neuroectoderm into the procephalic neuroectoderm were found to generate almost exclusively neural cell clones in the brain, suggesting that epidermalising signals in the procephalic neuroectoderm (as mediated by Notch signalling) are essentially absent (Stüttem and Campos-Ortega, 1991). Conversely, epidermal clones obtained upon transplantation of cells from the procephalic into the truncal neuroectoderm indicate that cells of the procephalic neuroectoderm are capable of responding to epidermalising signals mediated by cell-cell interactions (Stüttem and Campos-Ortega, 1991).

As a consequence of reduced lateral inhibition in the procephalic neuroectoderm, a high level of proneural gene expression would be maintained, allowing adjacent cells to develop as NBs. Similarly, in the truncal neuroectoderm of neurogenic mutants it has been shown that proneural gene expression does not become restricted to single cells, but instead all cells within proneural clusters show morphological characteristics and gene expression patterns of NBs (Lehmann et al., 1981; Martin-Bermudo et al., 1995; Seugnet et al., 1997; Skeath and Carroll, 1992; Stollewerk, 2000).

Interestingly, precursor formation in the midline region of the procephalic neuroectoderm, which gives rise to the stomatogastric nervous system (SNS), the visual system and medial parts of the brain, exhibits parallels. Like their mesectodermal counterparts in the trunk, the head midline cells do not give rise to typical NBs by delamination but remain integrated in the surface ectoderm and express proneural genes for an extended period of time (Hartenstein et al., 1996), except for an initial population of SNS precursors (Gonzalez-Gaitan and Jäckle, 1995). Dumstrei et al. (Dumstrei et al., 1998) have shown that genes involved in EGFR signalling are expressed in the head midline and proposed that the negative feedback loop between the concomitantly expressed proneural and neurogenic genes could be modified by EGFR signalling. This possibility was also raised in the context of cellular differentiation in the developing ommatidia (Schweitzer and Shilo, 1997). In anti-activated MAPK antibody staining (indicative of EGFR signaling) (Gabay et al., 1997), we find that activated MAPK is dynamically expressed in parts of the procephalic neuroectoderm from which brain NBs derive. For example, by stage 7, MAPK expression is found in mitotic domain B and slightly later in the neuroectoderm corresponding to domains 1, 2, 5 and 9 (R.U. and G.M.T., unpublished). This is compatible with the hypothesis that EGFR signaling inhibits Notch signaling in domain B (and possibly in other parts of the procephalic neuroectoderm) to enable neighbouring cells to delaminate as NBs, and thus produce a higher proportion of CNS progenitors when compared with the neuroectoderm of the trunk.

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