Development 130, 3621-3637

doi:10.1242/dev.00533

Molecular markers for identified neuroblasts in the developing brain of *Drosophila*

Rolf Urbach and Gerhard M. Technau

Institut für Genetik, Universität Mainz, D-55099 Mainz, Germany
Author for correspondence (e-mail: technau@mail.uni-mainz.de)

Accepted 4 April 2003

SUMMARY

The *Drosophila* brain develops from the procephalic neurogenic region of the ectoderm. About 100 neural precursor cells (neuroblasts) delaminate from this region on either side in a reproducible spatiotemporal pattern. We provide neuroblast maps from different stages of the early embryo (stages 9, 10 and 11, when the entire population of neuroblasts has formed), in which about 40 molecular markers representing the expression patterns of 34 different genes are linked to individual neuroblasts. In particular, we present a detailed description of the spatiotemporal patterns of expression in the procephalic neuroectoderm and in the neuroblast layer of the gap genes *empty spiracles*, *hunchback*, *huckebein*, *sloppy paired 1* and *tailless*; the homeotic gene *labial*; the early eye genes *dachshund*, *eyeless* and *twin of eyeless*; and several other marker genes (including *castor*, *pdm1*, *fasciclin 2*, *klumpfuss*, *ladybird*, *runt* and *unplugged*). We show that based on the combination of genes expressed, each brain neuroblast acquires a unique identity, and that it is possible to follow the fate of individual neuroblasts through early neurogenesis. Furthermore, despite the highly derived patterns of expression in the procephalic segments, the co-expression of specific molecular markers discloses the existence of serially homologous neuroblasts in neuromeres of the ventral nerve cord and the brain. Taking into consideration that all brain neuroblasts are now assigned to particular neuromeres and individually identified by their unique gene expression, and that the genes found to be expressed are likely candidates for controlling the development of the respective neuroblasts, our data provide a basic framework for studying the mechanisms leading to pattern and cell diversity in the *Drosophila* brain, and for addressing those mechanisms that make the brain different from the truncal CNS.

Key words: CNS, Brain development, Neuroblasts, Gap genes, Molecular markers, *Drosophila*

INTRODUCTION

The central nervous system (CNS) of *Drosophila*, which encompasses the brain and ventral nerve cord, develops from a bilateral neuroectoderm, which gives rise to multipotent neural stem cells, called neuroblasts (NB). Investigations of the mechanisms that control the development of NBs have so far mainly focused on the less complex ventral nerve cord. The ventral nerve cord has been shown to arise from a segmentally repeated pattern of NBs (Hartenstein and Campos-Ortega, 1984). Each NB acquires a unique identity, which is reflected by the time and position of its delamination from the neuroectoderm, by the combination of genes it expresses, and by the production of a specific cell lineage (Bossing et al., 1996; Broadus et al., 1995; Doe, 1992; Doe and Technau, 1993; Schmidt et al., 1997). Specification of a unique NB fate depends on positional information in the neuroectoderm, provided by the products of early patterning genes (reviewed by Bhat, 1999; Skeath, 1999). Experimental data suggest that once NBs are specified, their further development is largely controlled by their intrinsic properties (Brody and Odenwald, 2000; Prokop and Technau, 1994; Udolph et al., 1995). Such intrinsic properties might be established by the distinct combination of genes expressed in a NB (Doe, 1992; Isshiki et al., 2001).

To shed light on how neural diversity is generated in the *Drosophila* brain, it is of importance to know which genes (positional cues) are expressed within the procephalic neuroectoderm at different stages of development, and which genes are expressed in each individual brain NB. In the preceding papers, we have traced the pattern and modes of brain NB formation (Urbach et al., 2003), and assigned each NB to a particular brain neuromere by analysing the expression of segment polarity and dorsoventral patterning genes (Urbach and Technau, 2003). Here, we have analysed in detail the expression of the head gap genes *empty spiracles*, *hunchback*, *huckebein*, *sloppy paired 1* and *tailless*; the homeotic gene *labial*; and many other marker genes in the procephalic neuroectoderm, as well as in the brain NBs, until stage 11, when the full complement of NBs has evolved. In total, we provide an array of more than 40 molecular markers (antibodies, mRNA probes and enhancer trap lines) that represent the expression of 34 different genes. Most of these molecular markers are expressed in characteristic domains of the procephalic neuroectoderm, and all of them are

specifically expressed in particular subsets of brain NBs. Each brain NB forms at a stereotypical time and position (see also Urbach et al., 2003) and each exhibits a reproducible pattern of gene expression. Accordingly, it is now feasible to identify each brain NB uniquely based on its characteristic expression of certain molecular markers at different stages of early neurogenesis (stage 9-11). It is reasonable to assume that these marker genes are involved in the specification of brain NBs and components of their corresponding cell lineages. Knowing the individual precursor cells and their patterns of gene expression under normal conditions, is a prerequisite for the interpretation of mutant phenotypes, as well as the effects of experimental manipulations. Furthermore, the combination of molecular markers expressed in the identified NBs allows the identification of serially homologous NBs in the brain and ventral nerve cord. Thus, these comprehensive data are useful for investigating mechanisms leading to cell diversity in the developing embryonic brain, as well as those mechanisms which make the brain different from the truncal parts of the CNS.

MATERIALS AND METHODS

Drosophila strains

The following fly strains were used: Oregon R (wild type), *huckebein-lacZ* (5953) (Doe, 1992), *ming-lacZ* (1530) (Cui and Doe, 1992) (corresponds to *castor* expression) (Chiang et al., 1995), *unplugged-lacZ* (1912) (Doe, 1992) (*huckebein-*, *ming-* and *unplugged-lacZ* lines kindly provided by C. Q. Doe), *engrailed-lacZ* (ryXho25) (Hama et al., 1990), *klumpfuss-lacZ* (P212) (Yang et al., 1997) (kindly provided by X. Yang and B. Chia), *seven up-lacZ* (H162) (Mlodzik et al., 1990), *tailless-lacZ* (flies carrying the P1 construct) (Rudolph et al., 1997) (kindly provided by J. A. Lengyel).

Staging and mounting of embryos

Staging of the embryos was done according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997); additionally, we used the trunk NB pattern (Doe, 1992) as further morphological markers 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 N. Y. Yan), rabbit-anti-Castor (1:1000; kindly provided by W. Odenwald), mouse-anti-Dachshund (1:250) (Mardon et al., 1994) (Developmental Studies Hybridoma Bank), rabbit-anti-Deadpan (1:300) (Bier et al., 1992) (kindly provided by H. Vässin), anti-DIG-AP (1:1000, Roche), rat-anti-Empty spiracles (1:1000) (Walldorf and Gehring, 1992) (kindly provided by U. Walldorf), rabbit-anti-Eyeless (1:1000; kindly provided by U. Walldorf), mouse-anti-Fasciclin 2 (1D4, 1:15, Developmental Studies Hybridoma Bank), mouse-anti-β-Galactosidase (1:500, Promega), rabbit-anti-β-Galactosidase (1:2500, Cappel), rabbit-anti-Hunchback (1:1000, kindly provided by M. Gonzales-Gaitan), mouse-anti-Invected (4D9,1:4) (Patel et al., 1989) (Developmental Studies Hybridoma Bank), rabbit-anti-Klumpfuss (1:1000) (Yang et al., 1997) (kindly provided by X. Yang and B. Chia), rabbit anti-Labial (1:100) (Diederich et al., 1991) (kindly provided by T. Kaufman), mouse-anti-Ladybird (1:2) (Jagla et al., 1997) (kindly provided by K. Jagla), rat-anti-Orthodenticle (1:20) (Wieschaus et al., 1992) (kindly provided by S. Leuzinger and H. Reichert), rabbit-anti-POU-domain1 (1:500) (Yeo et al., 1995) (kindly provided by X. Yang and B. Chia), rabbit anti-Proboscipedia (1:200) (Cribbs et al., 1992) (kindly provided by D. Cribbs), rabbit-anti-Runt (1:500) (Dormand and Brand, 1998) (kindly provided by E. Dormand and A. Brand), rat-anti-Sloppy paired (1:300) (Cadigan et al., 1994b) (kindly provided by W. Gehring) and rabbit-anti-Twin of eyeless (1:400; kindly provided by U. Walldorf). 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

twin of eyeless (*toy*) and *huckebein* (*hkb*) RNA probes were synthesized using full-length cDNA of *toy* (Czerny et al., 1999) (kindly provided by T. Czerny and M. Busslinger) and *hkb* (kindly provided by G. Brönner); *eyeless* RNA probes were generated by PCR amplification using as a 5′primer 5′-CTTGGCTAAAGCTTTCATG-AGCAG-3′ and as a 3′primer 5′-TGAGTATTTAACAGCCGAAG-CTTC-3′; the resulting PCR fragment was used as a template. DIG-labelled RNA probes were prepared using a DIG-RNA-labelling mix (Roche) according to the manufacturers protocol. 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 AND DISCUSSION

The spatiotemporal pattern of gap gene expression in the procephalic neuroectoderm and brain neuroblasts

The cephalic gap genes are expressed in large domains of the procephalon and play a crucial role not only in the patterning of the peripheral ectoderm (for a review, see Jürgens and Hartenstein, 1993), but also in regionalizing the brain primordium (Hartmann et al., 2000; Hirth et al., 1995; Reichert, 2002; Younossi-Hartenstein et al., 1997). Our model of the segmental organization of the *Drosophila* brain is based on the expression pattern of segment polarity and DV patterning genes (see Urbach and Technau, 2003). To see whether the cephalic gap genes respect the neuromeric boundaries and to provide a basis for studying their potential role in the formation or specification of brain precursor cells we studied the expression of *orthodenticle*, *empty spiracles*, *sloppy paired 1*, *tailless*, *huckebein*, and *hunchback* in the developing head ectoderm, as well as in the entire population of identified NBs during stages 9-11 (Figs 1, 2).

In the cellular blastoderm *orthodenticle* (*otd*) is expressed in an anterior, circumferential stripe and subsequently fades in the ventral region to become restricted to the procephalic ectoderm after gastrulation (Finkelstein and Perrimon, 1990; Gao et al., 1996). In Otd/Engrailed (En) double labelling between stage 9 and 11, we find Otd expression in the pregnathal head to be confined to a large domain covering most of the antennal and preantennal (ocular) neuroectoderm [Fig. 1B,F, Fig. 2G,N; for a description of brain neuromers see Urbach and Technau (Urbach and Technau, 2003)]. Furthermore, Otd is detectable in all NBs delaminating from this domain (about 50 ocular and six antennal; Fig. 1A,E, Fig. 2H,M). NBs in the dorsal and

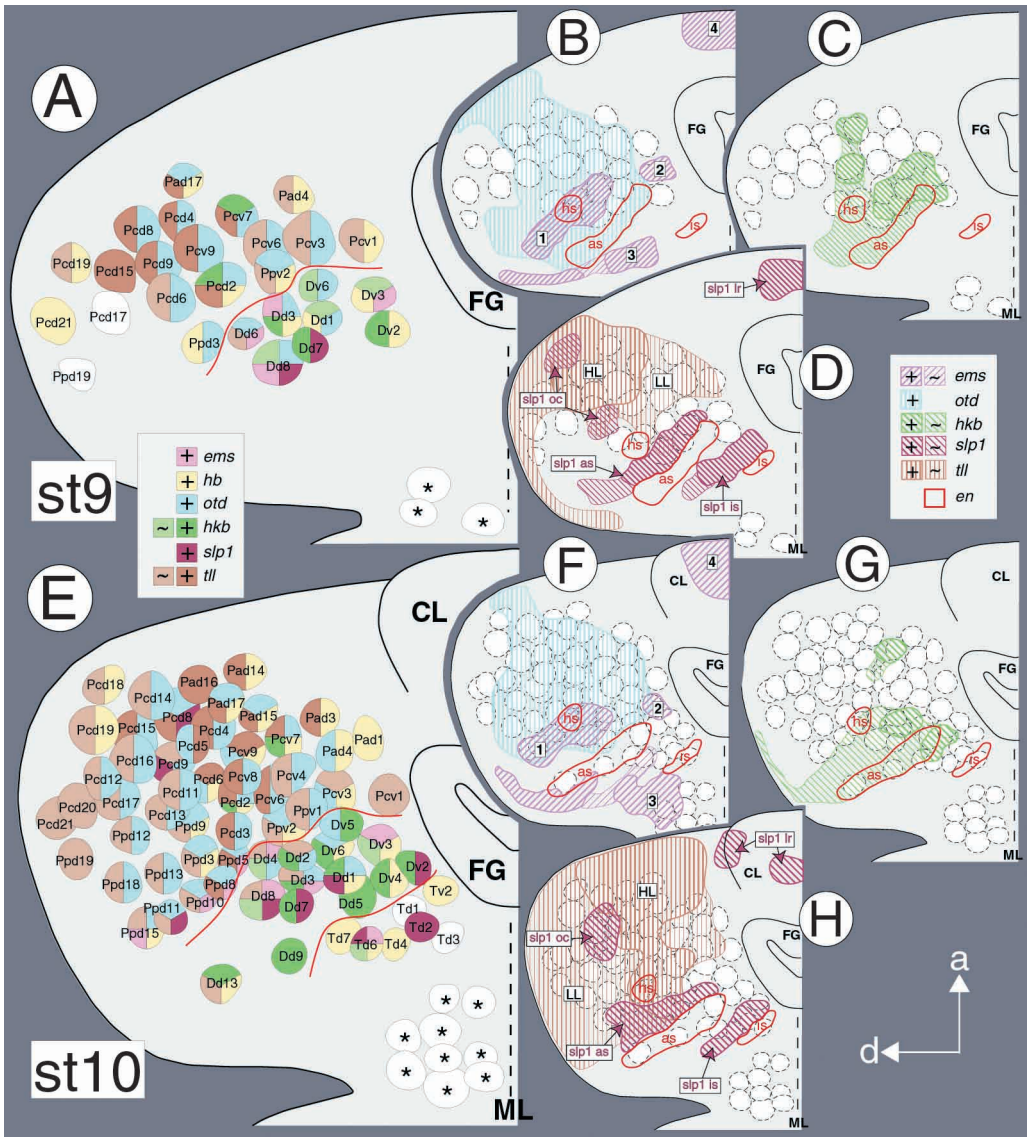

Fig. 1. Expression of gap genes at embryonic stages 9 (st9) and 10 (st10). Semi-schematic presentations of the left half of head flat preparations [anterior (a) is towards the top; dorsal (d) is towards the left]. Colour code indicates protein expression patterns of Empty spiracles (*ems*), Hunchback (*hb*), Orthodenticle (*otd*), Sloppy paired 1 (*slp1*), and the *huckebein-lacZ* (*hkb*) and *tailless-lacZ* (*tll*) patterns in identified brain neuroblasts [A,E; colour intensity reflects weak (~) and strong (+) expression] and the peripheral procephalic ectoderm (B-D; F-H; thick hatching indicates strong expression, thin hatching indicates weak expression) at stage 9 (A-D) and stage 10 (E-H). Nomenclature of brain NBs is according to their position in the trito- (T), deuto- (D) and protocerebrum [P; for details of the nomenclature see Urbach et al. (Urbach et al., 2003)]. Red lines in A,E mark the boundaries between trito-, deuto- and protocerebrum. In B,F, *ems* ectodermal domains arising from the blastodermal *ems* stripe are numbered (1-4; compare with Fig. 2N). In D,H, high level and low level *tll-lacZ* expression domains correspond to HL and LL in Rudolph et al. (Rudolph et al., 1997). Note, that *slp1* is segmentally expressed in the intercalary (slp1 is, *slp1* intercalary stripe) and antennal (slp1 as, *slp1* antennal stripe) ectoderm. Stars indicate mandibular NBs. Engrailed ectodermal domains (encircled in red) are shown for comparison. as, *en* antennal stripe; CL, clypeolabrum; FG, forgut; hs, *en* head spot; is, *en* intercalary stripe; ML, ventral midline; slp1 oc, ocular *slp1* domain; slp1 lr, labral *slp1* domain.

most anterior region of the protocerebrum are Otd-negative, including most NBs of the labral neuromere (Fig. 1E, Fig. 2M). Similar observations have been made by Younossi-Hartenstein et al. (Younossi-Hartenstein et al., 1997), that Otd covers the NBs of the anterodorsal part of the antennal segment and most of the acron (which is equivalent to the ocular segment). We also observed Otd expression in cells along the dorsal midline of the head, as well as faint expression in neuroectodermal cells in the ventral part of the intercalary segment (Fig. 2G,N), from which the weakly Otd-positive Tv1 emerges (Fig. 2M).

tailless (*tll*) has been shown to be expressed in an anterior horseshoe-shaped stripe in the cellular blastoderm, which after gastrulation shows a region of high ('HL domain') and a region of low level of *tll* expression ('LL domain'), and at stage 9 covers most of the protocerebral neuroectoderm (Rudolph et al., 1997) (see also Fig. 1D). Using a *tll-lacZ* line (Rudolph et al., 1997) at stage 9 we find *tll* expression in the developing brain in most protocerebral NBs (except the dorsoposterior ones; Fig. 1A). During stages 9-11 *tll-lacZ* expression expands in the protocerebral neuroectoderm beyond the En-positive head spot (hs; Fig. 1O,H, Fig. 2I,P). By stage 11 it is detectable in all protocerebral NBs (Fig. 2J,M), confirming earlier data (Rudolph et al., 1997; Younossi-Hartenstein et al., 1997). In addition, we find *tll-lacZ* in some ventral and dorsal deutocerebral NBs (Fig. 2J,M; Table 1), indicating that *tll* is not exclusively confined to protocerebral progenitors.

During early neurogenesis in the trunk, *empty spiracles* (*ems*) is metamerically expressed in lateral ectodermal patches. In the head, it acts as a gap gene, which is expressed in a circumferential procephalon domain in the early cellular blastoderm (Dalton et al., 1989; Walldorf and Gehring, 1992). We find that during gastrulation this circumferential stripe dissolves into three smaller ectodermal domains between the anterior part of the mandibular segment and the posterior part

Table 1. Combinatorial code of marker gene expression in identified brain neuroblasts

		Proneural				Gap						Segment polarity					DV			Early eye genes			Others									Glia	
		ac	*sc*	*l'sc*	*ato*	*ems*	*otd*	*tll*	*hb*	*slp1*	*hkb*	*hh*	*en*	*gsb*	*mirr*	*wg*	*msh*	*ind*	*vnd*	*dac*	*ey*	*toy*	*cas*	*fas II*	*lab*	*klu*	*libe*	*pdm*	*runt*	*syp*	*unpg*	*gcm*	*repo*
T	d1			•														•			•			•	•	•		•					
	d2			•						•				•				•			•		•	•	•	•		•					
	d3			•					•			•	•	•				•							•	•		•					
	d4			•					•					•		•	•						•		•	•	•	•					
	d5								•			•	•				•								•			•					
	d6			•		•			•	•	•						•							•	•	•		•		•			
	d7			•					•	•					•		•								•	•						•	•
	d8										•						•							•	•	•				•			
	v1						•				•													•	•	•		•					
	v2								•										•	•				•	•	•				•			
	v3			•						•				•					•						•								
	v4											•	•						•				•		•	•							
	v5											•	•		•				•						•			•					
D	d1			•				•		•	•	•			•	•		•					•					•	•	•	•		
	d2	•						•	•		•	•			•		•						•					•	•				
	d3	•		•		•	•	•			•	•					•					•						•	•	•	•		
	d4									•	•				•		•				•		•					•			•		
	d5										•	•	•	•			•		•				•					•	•		•		
	d6			•		•	•	•			•	•			•		•					•	•					•		•	•		
	d7			•						•	•	•		•		•	•						•			•	•	•		•	•		
	d8	•							•		•	•				•	•					•	•					•		•	•		
	d9										•	•	•				•							•							•		
	d10								•		•						•														•		
	d11				•							•					•							•							•		
	d12				•						•	•					•														•		
	d13		•		•				•	•		•	•				•													•	•		
	v1				•				•							•																	
	v2			•					•		•	•				•			•					•	•	•		•	•	•	•		
	v3				•	•	•	•	•	•	•	•				•							•			•		•		•	•		
	v4			•					•		•					•			•						•	•		•		•	•		
	v5			•						•	•	•				•										•		•	•	•	•		
	v6	•		•			•			•	•	•				•					•		•			•		•	•	•	•		
	v7					•					•					•					•		•			•		•	•	•	•		
	v8			•							•	•	•						•				•	•		•		•		•			
P	ad1	•	•	•					•	•						•												•		•			
	ad2						•		•		•															•		•					
	ad3		•	•					•	•			•													•			•				
	ad4	•	•				•		•	•			•		•				•							•		•					
	ad5		•	•					•				•										•					•					
	ad6		•						•				•										•			•		•					
	ad7		•	•					•				•								•					•		•		•			
	ad8			•					•		•		•										•			•		•		•			
	ad9			•					•				•										•			•		•					
	ad10		•	•					•				•										•										
	ad11								•														•			•			•				
	ad12								•																	•		•					
	ad13			•					•														•										
	ad14			•					•	•																•							
	ad15			•			•	•	•	•												•				•		•		•			
	ad16		•						•														•			•							
	ad17			•			•	•	•														•					•					
	ad18								•														•										
	av1			•					•	•			•							•		•	•			•		•		•			
	cd1								•	•						•				•		•				•		•					
	cd2			•					•	•	•									•	•	•	•			•		•		•			
	cd3	•	•	•					•	•						•						•	•			•		•					
	cd4								•	•										•		•	•			•		•		•			
	cd5			•					•	•												•				•		•					
	cd6	•		•					•	•	•					•				•		•	•			•		•		•			
	cd7								•	•	•					•		•				•	•			•							
	cd8	•		•					•	•	•									•			•			•		•	•	•			
	cd9	•		•					•	•	•									•		•				•		•	•	•			
	cd10			•					•	•						•							•					•	•				
	cd11								•	•						•						•	•			•		•	•				
	cd12								•	•	•					•						•	•			•		•	•				
	cd13								•	•	•					•		•				•	•			•		•					
	cd14			•					•	•										•	•	•	•					•					
	cd15	•		•					•	•													•			•			•				
	cd16	•		•					•	•						•							•						•				
	cd17			•					•	•						•						•	•			•		•	•				
	cd18									•						•						•	•						•	•			
	cd19	•		•						•	•					•						•	•			•			•				
	cd20		•	•						•												•	•					•	•				
	cd21	•	•	•						•	•												•			•		•		•			
	cv1			•						•						•			•				•			•		•					
	cv2			•				•	•							•			•				•			•							
	cv3	•		•				•	•	•						•			•				•					•		•			
	cv4			•				•	•		•		•						•				•			•		•			•		
	cv5			•		•	•	•											•				•			•		•					
	cv6			•				•	•	•	•								•		•	•	•			•		•		•	•		
	cv7	•	•	•				•	•	•	•								•	•	•	•	•			•		•		•			
	cv8			•				•	•	•									•				•			•	•						
	cv9	•		•				•	•	•									•	•	•	•	•			•		•		•	•		
	pd1		•					•	•		•					•				•		•	•			•					•		
	pd2					•	•	•				•			•							•	•			•		•					
	pd3			•				•	•					•		•						•	•			•			•	•			
	pd4							•	•							•						•	•			•		•					
	pd5			•		•	•	•				•	•									•	•			•		•					
	pd6							•	•							•		•				•	•			•							
	pd7							•	•							•						•	•			•							
	pd8			•		•	•	•				•	•							•		•	•			•		•					
	pd9			•				•	•							•		•				•	•			•		•	•	•			
	pd10			•		•	•	•				•			•							•	•			•							
	pd11	•	•	•				•	•		•	•								•			•			•					•		
	pd12							•	•							•					•	•	•			•		•	•				
	pd13			•				•	•							•		•				•	•			•			•				
	pd14						•	•														•	•			•					•		
	pd15		•	•		•	•	•	•	•		•											•			•					•		
	pd16		•					•	•	•		•											•					•			•		
	pd17		•	•					•														•	•					•				
	pd18			•				•	•			•									•	•	•			•				•	•		
	pd19				•				•													•	•										
	pd20							•	•			•									•	•				•				•	•		
	pv1			•				•	•		•								•				•			•		•	•				
	pv2	•		•				•	•	•		•							•		•	•				•	•	•		•			
	pv3			•				•	•		•								•			•	•	•	•	•	•			•			

T, tritocerebral neuroblasts; D, deutocerebral neuroblasts; P, protocerebral neuroblasts.
Expression of genes indicated at the top includes stages 9, 10 and 11.

of the ocular segment (1-3 in Fig. 1B,F), which are not in segmental register. During further development, the third domain splits into a mandibular/intercalary and an antennal component (3a and 3b in Fig. 2N). All these domains contribute NBs to the brain (Fig. 1A,E, Fig. 2A,B,M). In addition to *ems* expression in the intercalary and antennal segments, and the corresponding trito- and deutocerebral neuromers (see also Hartmann et al., 2000; Hirth et al., 1995; Younossi-Hartenstein et al., 1997), we also identified *ems* expression in a small neuroectodermal region (5 in Fig. 2N) and NBs of the ocular segment (Fig. 2A,B,M). Finally, a further *ems* patch is located in the dorsoanterior procephalic ectoderm ['dorsal patch' according to Walldorf and Gehring (Walldorf and Gehring, 1992)], which becomes part of the labral ectoderm and does not appear to give rise to brain NBs (4 in Fig. 1B,F, Fig. 2N). Ems/En double labelling confirm the segmental affiliation of the Ems-positive ectodermal regions described above. Thus, from stage 9 onwards, part of the antennal/ocular *ems* domain (1 in Fig. 1B,F, Fig. 2N) overlaps with the En-positive hs (Fig. 1B), and from stage 10/11 onwards these genes are also found to be co-expressed in the en hs-derived protocerebral NBs Ppd5 and Ppd8 (Fig. 1E, Fig. 2B,M; although *en* and *ems* expression also partly overlaps in the trunk ectoderm, we never observed a co-expression of both genes in trunk NBs; data not shown). In contrast to earlier observations, showing that most of the tritocerebral NBs are included in the *ems*-expressing domain (Younossi-Hartenstein et al., 1997), we identified only the dorsal Td6 to be Ems-positive (Fig. 1E, Fig. 2B,M). Ems protein is detectable in clusters of brain cells until the end of embryogenesis.

The *sloppy paired* (*slp*) locus contains the two related genes *slp1* and *slp2* (Grossniklaus et al., 1992). *slp1*, which acts as a head gap gene, plays a predominant role in head formation, while *slp2* is largely dispensable (Cadigan et al., 1994a; Grossniklaus et al., 1994). In the trunk neuroectoderm, where *slp1* has a function as a pair-rule and segment polarity gene, it is segmentally expressed in neuroectodermal stripes (Cadigan et al., 1994a; Cadigan et al., 1994b) as well as in NBs of row 4 and 5 (Bhat et al., 2000). We find this segmental appearance of *slp1* expression to be conserved in parts of the procephalon. In the blastoderm, Slp1 protein is detected in a large domain of the procephalon anlage, which subsequently diminishes in its anterior/ventral part (data not shown). As a result, only the posterior half of the original *slp1* domain remains as a circumferential ring ['head stripe' according to Grossniklaus et al. (Grossniklaus et al., 1994)] and gets separated from the anterodorsal part ['head cap' according to Grossniklaus et al. (Grossniklaus et al., 1992)]. To follow the dynamics in the Slp1

expression pattern, we examined Slp1/En double labelling during stages 8-11. We find that the 'head stripe' corresponds to the *slp1* stripe of the prospective mandibular segment (data not shown), and the posterior part of the 'head cap' to the Slp1-positive stripe in the prospective antennal segment (slp1 as; Fig. 1D). At the beginning of gastrulation, a new Slp1 ectodermal spot in the anterodorsal procephalon is observed (Grossniklaus et al., 1992); this spot later becomes part of the labral ectoderm (slp1 lr; Fig. 1D). In addition, at stage 9, three new ectodermal domains become detectable: one stripe anterior to the *en* intercalary stripe belonging to the intercalary segment [slp 1 is, Fig. 1D; 'hypopharyngeal stripe' according to (Grossniklaus et al., 1992)], and two small spots in the region of the ocular segment (anterior to the *en* head spot; slp1 oc, Fig. 1D). Except for the labral domain, the *slp1* domains contribute NBs to the brain (Fig. 1A,E, Fig. 2M,L; Table 1). Thus, *slp1* is segmentally expressed in the procephalic neuroectoderm and subsets of brain NBs, resembling the situation in the trunk (Bhat et al., 2000; Cadigan et al., 1994a; Cadigan et al., 1994b). At stage 11 patchy expression of Slp1 becomes detectable within the ocular and labral ectoderm (Fig. 2K,P) and in some underlying ocular and labral NBs (Fig. 2M). Some of these NBs initiate *slp1* expression after delamination; e.g. Pcv6 and Pcd2 delaminate at stage 9 and do not express *slp1* before stage 11 (compare Fig. 1A with Fig. 2M). Slp1 expression is observed in the brain until the end of embryogenesis (data not shown).

huckebein (*hkb*), a terminal gap gene, is first expressed at the anterior and posterior blastodermal poles, where it is required for the specification of the endodermal anlagen, and later for the invagination of the stomodeum (Brönner and Jäckle, 1991; Weigel et al., 1990). After gastrulation, *hkb* becomes transiently expressed in a repetitive pattern in the trunk neuroectoderm and in eight, mainly intermediate, NBs per hemineuromere (Broadus et al., 1995; McDonald and Doe, 1997). In the procephalic region at the cellular blastoderm stage, we find *hkb* expression in a centrally located stripe and a dorsal ectodermal spot (data not shown). *hkb* in situ hybridization combined with anti-Inv antibody staining reveals that during stage 9/10 the hkb stripe covers most of the antennal ectoderm and reaches into the anterior region of the intercalary segment, and the hkb spot covers part of the ocular ectoderm (Fig. 1C,G). During stage 9, *hkb* transcript in the ocular spot becomes progressively restricted to the delaminating protocerebral NBs, Pcv7 and Pcd2 (Fig. 1A,E), and remains strongly expressed in both NBs until stage 11 (Fig. 2M). In the antennal domain during stage 10/11 the transcript becomes confined to three to five deutocerebral NBs. However, using a *hkb-lacZ* line (5953) (Broadus et al., 1995) the marker is expressed in all deutocerebral NBs at stage 10 (Fig. 1E). At stage 11, *hkb-lacZ* was not detectable in Dd8 and Dd11, indicating that *hkb* is not a general deutocerebral NB marker (Fig. 2M). In the tritocerebrum, *hkb* is expressed only in Td6 (stage 10; Fig. 1E) and in Tv1, Td8 (stage 11; Fig. 2M). Thus, although expressed in a few trito- and protocerebral NBs, *hkb* expression appears to be mainly confined to the antennal neuroectoderm and NBs. Compared with the transcript, which becomes restricted to the NBs during stage 9-11, *hkb-lacZ* expression has a longer perdurance in the peripheral ectoderm and corresponding NBs. By stage 14, most of the *hkb* transcript has disappeared and is confined to some deutocerebral cells; *hkb-lacZ* is strongly expressed until the end of embryogenesis in deutocerebral, and at a lower level, in protocerebral cells, the putative progeny of the identified Hkb-positive brain NBs (data not shown).

hunchback (*hb*) expression in the anterior half of the embryo falls below the limit of detection at the beginning of germ band extension, but accumulates during the extended germ band stage in the CNS (Tautz et al., 1987), where it is transiently expressed in early, fully delaminated, trunk NBs (S1 and S2) and their progeny (Jimenez and Campos-Ortega, 1990; Kambadur et al., 1998). Antibody staining reveals that, from stage 8 onwards, Hb protein is not detected in the head neuroectoderm, but is very dynamically expressed in brain NBs. At stage 9, only about half of the identified deuto- and protocerebral NBs show Hb protein at a detectable level (Fig. 1A), suggesting that Hb is not a general marker for early NBs. Correspondingly, we find that Hb protein is also lacking in particular S1 and S2 NBs of the trunk (data not shown). In some of the early brain NBs, Hb first becomes detectable at stage 10, after their delamination. For example, the early NBs Pcv9 and Pcd6 delaminate at late stage 8 but do not start Hb expression before stage 10 (Fig. 1A,E). By stage 10, Hb is expressed in about 26 brain NBs, most of which delaminate between stage 9 and 10 (Fig. 1E). In most of these NBs, Hb expression is progressively lost, but is observed in an increasing amount of progeny cells. At stage 11, it is confined to a small subpopulation of about five tritocerebral and four to six protocerebral NBs (Fig. 2C,D,M). Thus, as opposed to the trunk, *hb* expression in the brain is not limited to early NBs. Hb is expressed in the brain until stage 15, when it is detected in a few cells of the protocerebrum (data not shown).

Taken together, among the cephalic gap genes, *slp1* appears to respect segmental boundaries during early neurogenesis of the brain. By contrast, we find that in the considered period of development (stage 9-11), the expression of *ems*, *otd* and *tll* does not seem to respect these borders, which has been claimed in previous reports (for *otd* and *ems*) (Hirth et al., 1995) (for *tll*) (Younossi-Hartenstein et al., 1997). All three genes are expressed in NBs deriving from ectodermal domains that are part of two or three neighbouring segments. For example, *ems* is expressed in a small number of NBs comprising about six posterior ocular and four anterior deutocerebral NBs (all of which derive from the same *ems* domain, except Dv3 and Pcv5), and one tritocerebral NB. Accordingly, *ems* mutants show defects in the intercalary, antennal (Hirth et al., 1995; Younossi-Hartenstein et al., 1997), and the ocular segment (e.g. the en hs is missing) (Schmidt-Ott et al., 1994). Considering that *ems* is expressed in only a few trito- and deutocerebral NBs it is remarkable that *ems* mutants show a deletion of the trito- and deutocerebrum (Hirth et al., 1995). An explanation for this could be that *ems* expression, which during earlier development covers the neuroectoderm of the respective segments, possibly confers specific identities to the arising trito- and deutocerebral NBs. The lack of these NBs might be responsible for the loss of NB-specific gene expression (Hartmann et al., 2000), and (secondarily) for the gross morphological defects seen in the *ems* mutant brain (Hirth et al., 1995). A similar proposal has been made to explain the brain defects that occur in *buttonhead* (*btd*) mutants, although *btd* is not expressed in NBs of the corresponding brain regions (Younossi-Hartenstein et al., 1997).

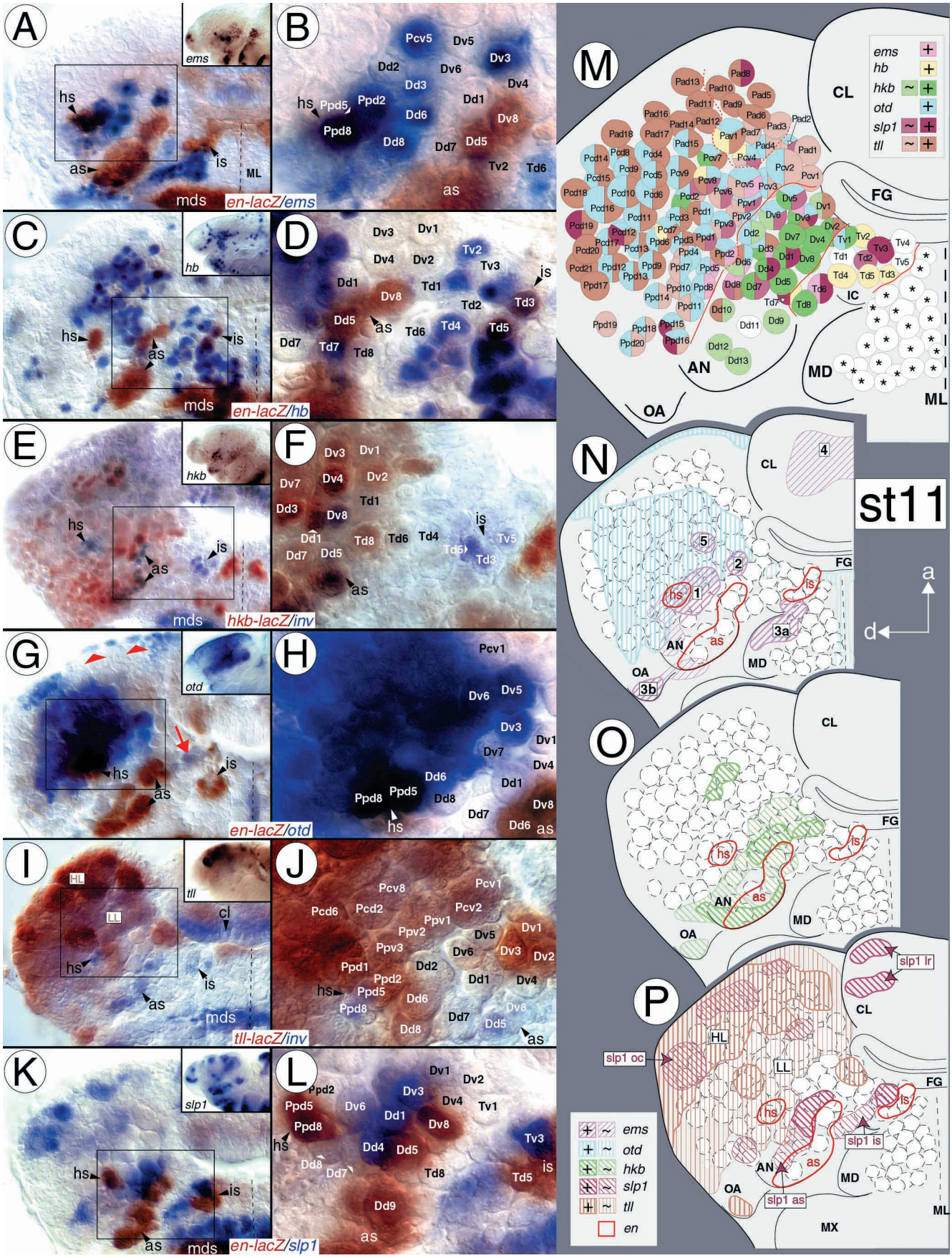
A
ems
hs
as
is
ML
mds
en-lacZ/ems
B
Pcv5
Dv5
Dv3
Dd2
Dv6
Dd3
Dv4
Dd1
Dd6
Dv8
Ppd8
Dd8
Dd7
Dd5
Tv2
Td6
C
hb
mds
en-lacZ/hb
D
Dv1
Tv2
Tv3
Td1
Dv8
Td2
Td3
Dd5
Td4
Td5
Dd7
Td7
Td8
E
hkb
mds
hkb-lacZ/inv
F
Dv7
Dv4
Dv2
Td1
Dd3
Dd1
Td8
Td6
Td4
Tv5
Td3
Dd7
Dd5
G
otd
en-lacZ/otd
H
Pcv1
Dv6
Dv5
Dv3
Dv7
Dd6
Ppd8
Ppd5
Dd8
Dd7
Dd6
I
HL
LL
cl
tll
mds
tll-lacZ/inv
J
Pcv8
Pcv1
Pcd6
Pcd2
Pcv2
Ppv1
Ppv2
Dv5
Dv1
Ppv3
Dv6
Dv3
Dv2
Ppd1
Dd2
Ppd2
Dd1
Dv4
Ppd5
Dd6
Ppd8
Dd7
Dd5
Dv8
Dd8
K
slp1
mds
en-lacZ/slp1
L
Dv1
Dv2
Ppd2
Ppd5
Dv3
Dv6
Dv4
Tv1
Ppd8
Dd1
Dv8
Tv3
Dd4
Dd5
Dd8
Dd7
Td8
Td5
Dd9
M
CL
FG
IC
MD
ML
AN
OA
ems
hb
hkb
otd
slp1
tll
N
st11
CL
4
5
2
FG
1
3a
AN
MD
OA
3b
a
d
O
CL
FG
AN
MD
OA
P
slp1 lr
CL
slp1 oc
HL
LL
FG
slp1 is
AN
MD
OA
slp1 as
ML
MX
ems
otd
hkb
slp1
tll
en

Fig. 2. Expression of gap genes at embryonic stage 11 (st11). (A-L) Left half of head flat preparations double stained for expression of a gap gene and *en/inv* (*lacZ* or antibody staining) as indicated. Pictures on the left (A,C,E,G,I,K) focus on the peripheral procephalic ectoderm; broken lines indicate the ventral midline; insets depict lateral views of stage 11 whole-mount heads. Middle panels (B,D,F,H,J,L) show close-up views of regions indicated in A,C,E,G,I,K by black frames; the focus is at the level of NBs. Positively stained NBs are indicated by white labels, negative ones are indicated by black labels. (A,B) Ems/*en-lacZ*. (C,D) Hb/*en-lacZ*. (E,F) *hkb-lacZ*/Inv. (G,H) Otd/*en-lacZ*. Note that Otd is expressed in the ventral midline, in a small domain within the ventral intercalary ectoderm (red arrow) and in cells along the dorsal midline (red arrowheads). (I,J) *tll-lacZ*/Inv; high level (HL) and low level (LL) *tll-lacZ*-expression is indicated. (K,L) Slp1/*en-lacZ*. (M) Summary of gap gene expression in brain NBs as indicated by the colour code, which distinguishes between weak (~) and strong (+) expression of *tll*- and *hkb-lacZ*, and Slp1. Red lines mark the boundaries between tritocerebrum, deutocerebrum, ocular and putative labral neuromer. Stars mark mandibular NBs. (N-P) Semi-schematic presentation of gap gene expression in the peripheral procephalic ectoderm. (N) *ems* ectodermal domains are numbered (1-5), 3a and 3b derive from intercalary *ems* domain 3 (compare with Fig. 1B,F). Anterior (a) is towards the top; dorsal (d) is towards the left. AN, IC, MD, MX, antennal, intercalary, mandibular and maxillary segment, respectively; CL, clypeolabrum; FG, foregut; ML, ventral midline; OA, Bolwig organ/optic lobe anlagen; as, *en* antennal stripe; cl, *en* expression in the clypeolabrum; hs, *en* head spot; is, *en* intercalary stripe; mds, *en* mandibular stripe; slp1 as, *slp1* antennal stripe; slp1 is, *slp1* intercalary stripe; slp1 oc, ocular *slp1* domain; slp1 lr, labral *slp1* domain.

Expression of the homeotic gene *labial* completely covers the anlage of the tritocerebrum

We analysed the expression of the homeotic genes *proboscipedia* (*pb*) and *labial* (*lab*), both members of the ANT-C and known to be expressed in the head ectoderm and in the brain after mid-embryogenesis (Hirth et al., 1998; Pultz et al., 1988). Antibody staining against Pb reveals that at stage 11, the protein is restricted to internal cells of the mandibular segment (presumably mesodermal cells) and to dorsal ectoderm of the maxillary and labial appendages (Pultz et al., 1988) (data not shown). We did not detect Pb protein in brain NBs.

lab was described to be expressed in the posterior tritocerebrum at stage 14 (Hirth et al., 1998). Using an antibody, we investigated the expression of Lab protein during early neurogenesis. From stage 9 onwards, Lab is detected in the ectoderm of the intercalary segment, and presumably in a small part of the posteroventral antennal segment (Fig. 3B,D,F). At that stage, the only NB expressing Lab protein is Dv2 (Fig. 3A). Double labelling against En reveal that at stage 11 Lab is expressed throughout the ectoderm of the intercalary segment (Fig. 3F,G), supporting previous reports (Diederich et al., 1989; Diederich et al., 1991; Mahaffey et al., 1989). The Lab domain overlaps posteriorly with the *en* intercalary stripe (en is), indicating that posterior borders of *lab* expression and of the intercalary segment coincide (Fig. 3F,G). The character of the anterior border of the *lab* domain is less clear. Dorsally, it runs along the posterior border of the *en* antennal stripe (en as); ventrally, however, it reaches the anterior border of the en as. This suggests, that the anterior border of the *lab* domain is segmental in the dorsal region and parasegmental in the ventral region (Fig. 3F,G). Interestingly, also for *scr* and *dfd*, which are other members of the ANT-C, it was reported that they initiate expression in a jagged stripe resolving into a pattern that is dorsally segmental and ventrally parasegmental (Gorman and Kaufman, 1995; Rogers and Kaufman, 1996). All NBs arising from the Lab-positive neuroectoderm express *lab*, among them all tritocerebral NBs and two ventral NBs, which we attributed to the deutocerebrum (Dv2 and Dv4) because they are located on the same anteroposterior level as the *en*-expressing Dv8 and Dd5 (Fig. 3A,C,E,H; Table 1) (see also Urbach and Technau, 2003).

Expression of the early eye genes *dachshund*, *eyeless* and *twin of eyeless*

dachshund (*dac*) was shown to be involved in the development of the eye (Mardon et al., 1994; Shen and Mardon, 1997) and the mushroom bodies (Kurusu et al., 2000; Martini et al., 2000) where it is expressed already in the progenitor cells (Noveen et al., 2000). Using an antibody, we find Dac expression in the trunk CNS not before stage 12; it is expressed in only two or three cells (not NBs) per neuromer (data not shown). In the procephalon, Dac is already detected by stage 9 in a small area of the dorsal ocular neuroectoderm (Fig. 4D) from which four Dac-positive NBs (Pcd4, Pcd8, Pcd9, Pcv9) delaminate (Fig. 4A). It has been suggested that the NBs delaminating from this Dac domain represent the progenitors of the mushroom body and co-express *eyeless* (*ey*) (Noveen et al., 2000). In disagreement with this, we find that, at that stage, the co-expression of both genes is confined only to a small region of the Dac-positive neuroectoderm (Fig. 4D) and to only one of the four identified Dac-positive NBs. As evidenced by Dac/Ey antibody double labelling, this NB (Pcv9) is one of the five Ey-positive brain NBs identified at stage 9 (Fig. 4A) (data not shown). Until stage 11, the Dac-expressing ocular domain expands into the antennal segment and into the optic lobe anlage [Fig. 4E-G; now encompassing also the ectodermal region called ‘para-MB neuroectoderm’ according to Noveen et al. (Noveen et al., 2000)], and a further spot appears in the clypeolabral ectoderm (Fig. 4E-G). At this stage, Dac protein can be observed in 13 protocerebral NBs and in the tritocerebral Tv2, but in no deutocerebral NBs (Fig. 4C,G,H). From stage 12 onwards, Dac becomes expressed in an increasing number of scattered cell clusters in the brain and ventral nerve cord (data not shown).

eyeless (*ey*), which encodes a member of the *Pax6* family of transcription factors, is a crucial regulator for eye development (Halder et al., 1995). It is expressed in the embryonic ventral nerve cord and brain (Kammermeier et al., 2001; Quiring et al., 1994) and has been shown to be involved in the development of the mushroom bodies (Kurusu et al., 2000; Noveen et al., 2000). Although it has been suggested that *ey* is expressed in the progenitor cells of the mushroom bodies (Noveen et al., 2000), *ey* expression in the evolving NB pattern of the ventral nerve cord and brain has not been described so far. Using an Ey antibody (which principally shows the same pattern as *ey* mRNA in situ hybridization; data not shown), we find Ey in the trunk to be expressed in segmentally reiterated ectodermal stripes and, at stage 11, in six NBs per hemineuromere (data not shown). In the posterior pregnathal head segments (intercalary and antennal), a segmental

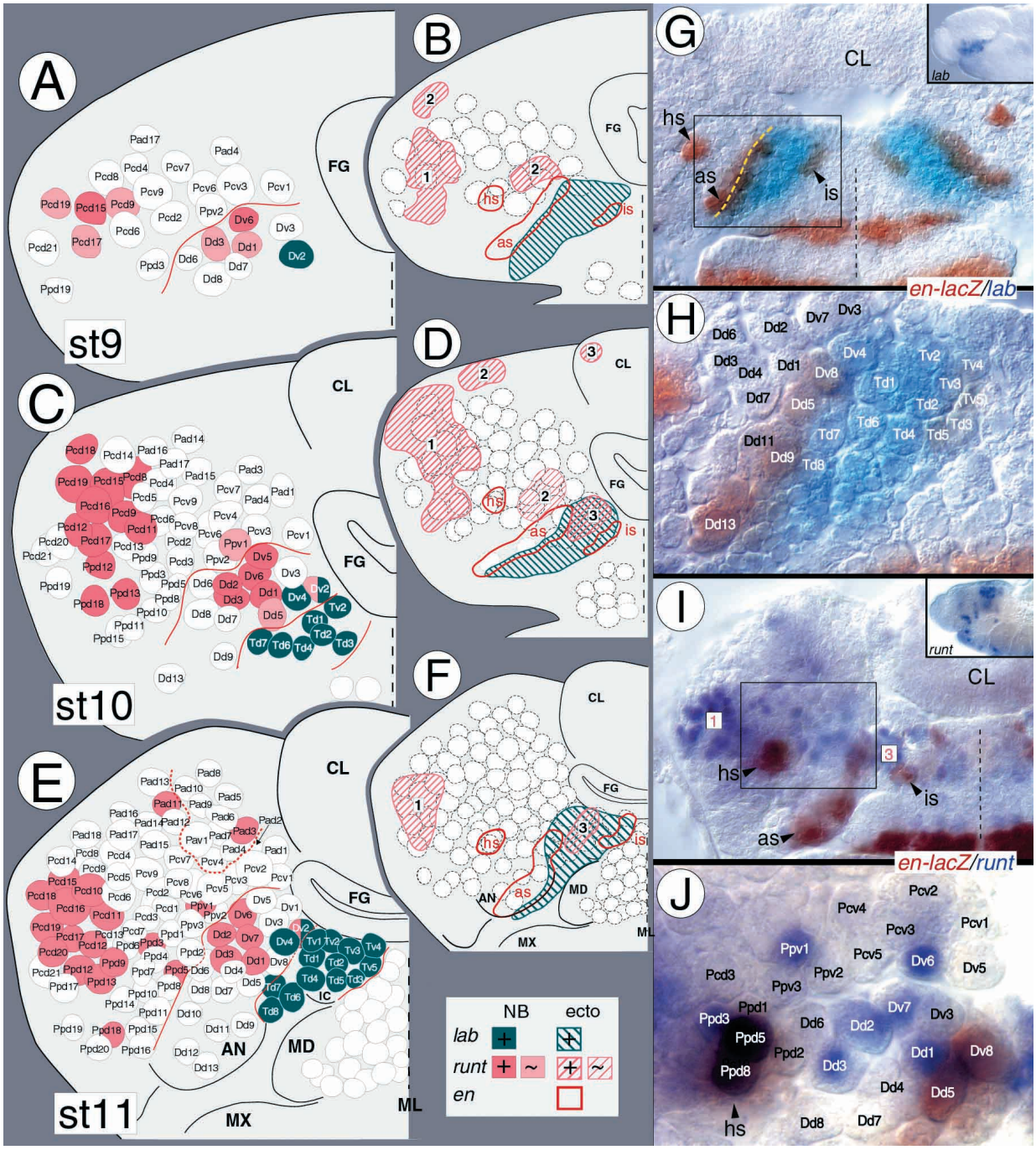

Fig. 3. Expression of the pair rule gene *runt* and the homeotic gene *labial* at embryonic stages 9, 10 and 11. Semi-schematic presentation of the Runt and Labial protein (Lab) patterns in brain NBs (A,C,E) and outer procephalic ectoderm in relation to the *en* pattern (B,D,F) at stage 9 (st9, A,B), stage 10 (st10, C,F) and stage 11 (st11, E,F) as indicated; numbers (1-3) in B,D,F reflect the temporal sequence of the ectodermal *runt* domains. Lab expression covers the complete intercalary neuroectoderm and emerging NBs. (E) Note that all trito- and two deutocerebral NBs express Lab; red lines demarcate neuromeric boundaries. (G,H) *en-lacZ*/Lab double stained head flat preparation. (G) Lab expression covers the *en* intercalary stripe (is) and partially the *en* antennal stripe (as; compare with F); broken line marks the anterior boundary of the Lab domain. (H) Higher magnification of the region indicated by black frame (in G) focusing at the level of NBs. Note that the Lab-positive Dv4 is at the same anteroposterior level as the En-positive Dv8. (I,J) *en-lacZ*/Runt double labelled head flat preparation. (I) In the periphery, Runt expression is confined to the ocular (1) and intercalary (3) ectodermal domain. (J) Higher magnification of region marked by black frame in I, focusing at the level of NBs. Positively stained NBs in H,J are indicated by white labels, negative ones are indicated by black labels; insets in G,I show a lateral view of stage 11 wholemounts; dashed line marks the ventral midline. Anterior (a) is towards the top; dorsal (d) is towards the left. AN, IC, MD, MX, antennal, intercalary, mandibular and maxillary segment, respectively; CL, clypeolabrum; FG, foregut; ML, ventral midline; as, *en* antennal stripe; hs, *en* head spot; is, *en* intercalary stripe.

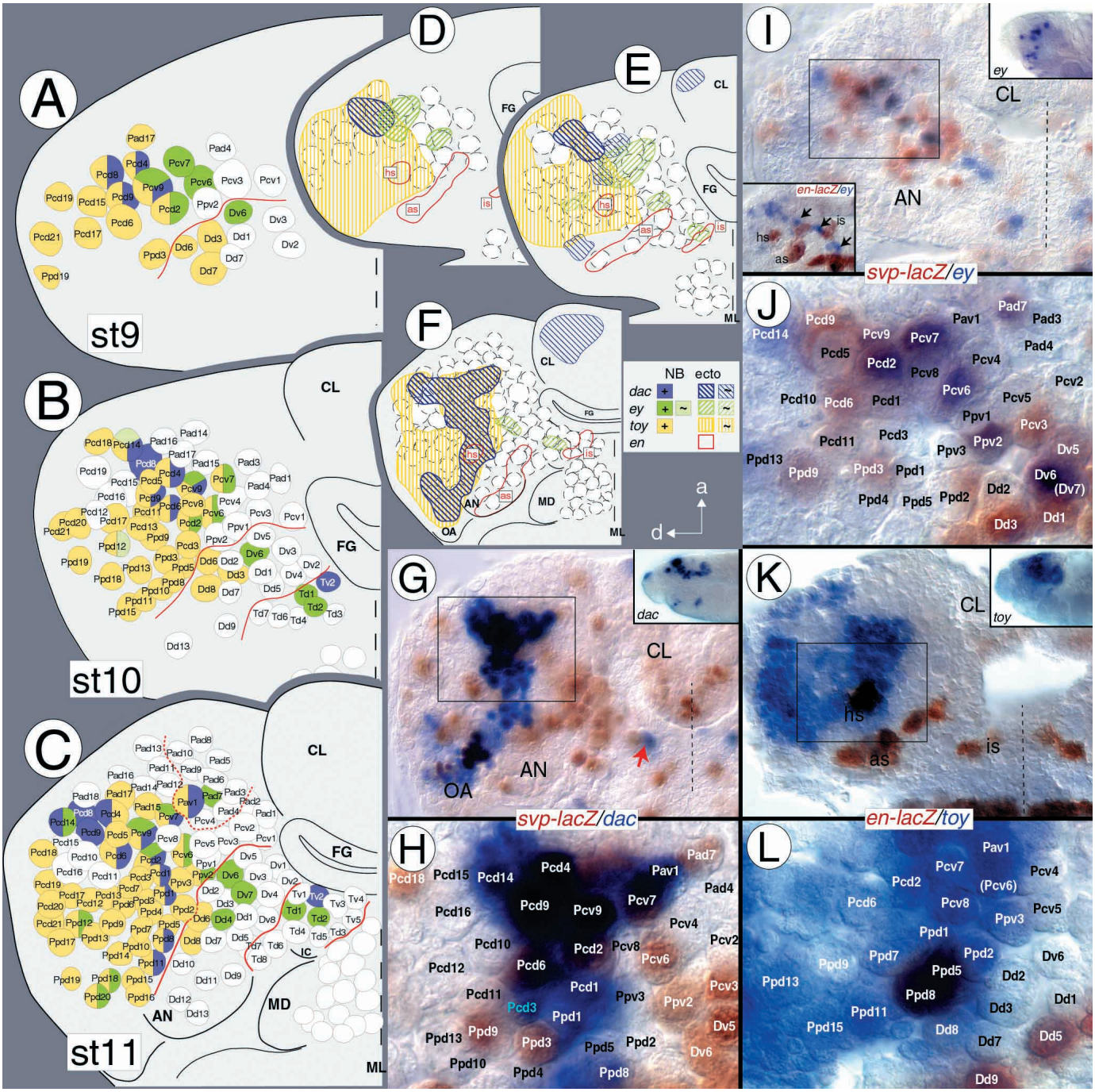

Fig. 4. Expression of the early eye genes *dac*, *ey* and *toy* at embryonic stages 9, 10 and 11. Semi-schematic presentation of Dachshund (*dac*), Eyeless (*ey*), and Twin of eyeless (*toy*) expression in brain NBs [A-C; low (~) and high (+) expression levels; red lines indicate neuromeric boundaries], and within the outer procephalic ectoderm (D-F; thick hatching indicates strong expression, thin hatching indicates weak expression) as indicated at stage 9 (st9, A,D), stage 10 (st10, B,E), and stage 11 (st11, C,F). Engrailed (*en*) expression domains in the peripheral ectoderm are indicated for comparison. (G-L) Double staining for expression of *svp-lacZ* (G-J) or *en-lacZ* (K,L), and Dachshund (*dac*; G,H), Eyeless (*ey*; I,J) and Twin of eyeless (*toy*; K,L). (G,I,K) Outer procephalic ectoderm of flat preparations (dashed line indicates ventral midline); insets show lateral views of stage 11 wholemounts. (H,J,L) Higher magnifications of regions indicated by frames in G,I,K at the level of brain NBs. (G) Red arrow indicates the intercalary Dac-positive ectodermal domain including the Dac-positive NB Tv2. (I,J) Ey is expressed in few NBs; Ey/*en-lacZ* double labelling (see inset on bottom left in I) shows Ey expression in the intercalary and antennal head immediately anterior to the respective *en* domains (arrows). (K,L) Toy is mainly confined to protocerebral NBs and to Dd8. Anterior (a) is towards the top; dorsal (d) is towards the left (except for insets). 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; hs, *en* head spot; is, *en* intercalary stripe.

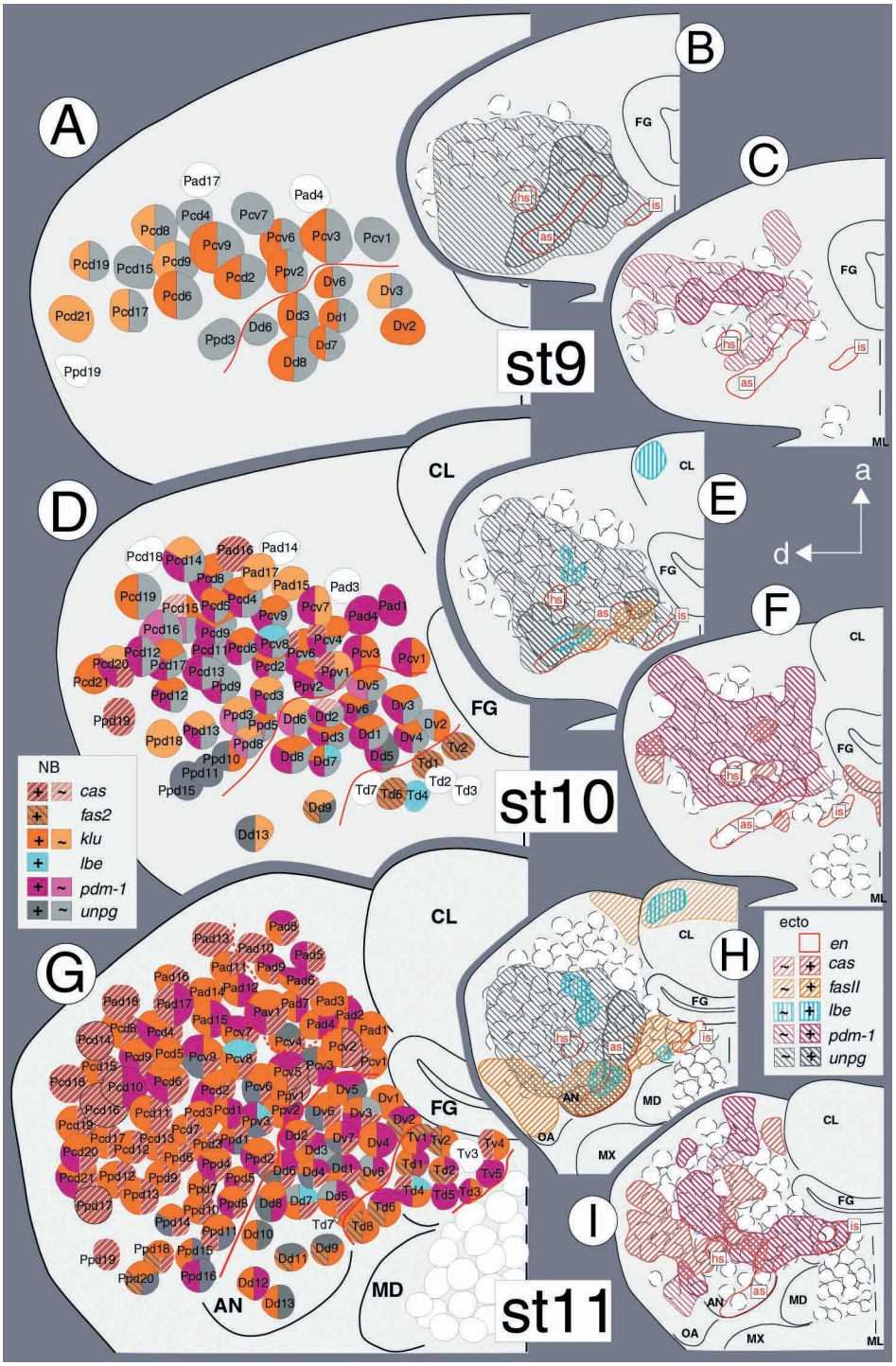

Fig. 5. Expression of *cas*, *Fas2*, *klu*, *lbe*, *pdm1* and *unpg* at embryonic stages 9, 10 and 11. Semi-schematic presentation of Castor (*cas*), Fasciclin 2 (*Fas2*), Klumpfuss (*klu*), Ladybird early (*lbe*), POU-domain 1 (*pdm1*) protein, and *unplugged-lacZ* (*unpg*) expression in brain NBs [A,D,G; low (~) and high (+) expression levels; red lines mark neuromeric boundaries], and within the outer procephalic ectoderm [B,C,E,F,H,I; thick hatching indicates strong (+), thin hatching weak (~) expression] as indicated at stage 9 (st9, A-C), stage 10 (st10, D-F) and stage 11 (st11, G-I). Engrailed (*en*) expression domains in the peripheral ectoderm are indicated for comparison. Anterior (a) is towards the top; dorsal (d) is towards the left. AN, MD, MX, antennal, mandibular and maxillary segment, respectively; CL, clypeolabrum; FG, foregut; ML, ventral midline; OA, Bolwig organ/optic lobe anlagen; as, *en* antennal stripe; hs, *en* head spot; is, *en* intercalary stripe.

expression of Ey appears to be fundamentally conserved. Ey/En double labelling reveals that the Ey-positive spots (and emerging NBs) in both segments are localized just anterior to their En-positive counterparts [Fig. 4F,I (lower inset); Fig. 7]. We detected Ey protein by stage 9 in five NBs: Pcv6, Pcv7, Pcv9, Pcd2 (which derive from an ocular ectodermal Ey domain) and Dv6 (which develops from a small ectodermal Ey spot in the antennal segment) (Fig. 4A,D). By stage 10, *ey* becomes expressed in an intercalary ectodermal spot, from which, by that stage, the Ey-positive Td2 and (slightly later) Td1 delaminate, and in a second ocular ectodermal spot from which Ppd12 develops (Fig. 4B,E). In contrast to the intercalary and antennal ectodermal spot, Ey protein becomes depleted during stage 11 in the ocular domain (Fig. 4F,I). At late stage 11, Ey is found in 15 brain NBs, including two intercalary, three deutocerebral, nine ocular and one labral NB (Pad7; Fig. 4C,J). During subsequent development of the brain, the Ey expression pattern becomes complex, especially in the preantennal segments, but it appears to be mainly confined to the progeny of the identified NBs (data not shown).

The second *Drosophila Pax6* gene, *twin of eyeless* (*toy*), has been shown to be expressed in the blastoderm in an anterodorsal patch that represents the posterior region of the procephalon anlage, and later in the embryonic brain (including the mushroom body; Kurusu et al., 2000) and visual system (Czerny et al., 1999). At stage 9, Toy expression encompasses the dorsal ocular and the anterodorsal part of the antennal ectoderm (as confirmed by double labelling against Toy and En; Fig. 4D). All NBs that delaminate from this part of the neuroectoderm express Toy (Fig. 4A-C). By stage 11, these include about 40 protocerebral NBs, all of which derive from the ocular ectoderm, except the putative labral Pav1, and two deutocerebral NBs (Fig. 4B,C,E,F,K,L). This pattern of expression in NBs closely matches the pattern that has been observed for *otd* (see *otd* section and Fig. 7). In some of these

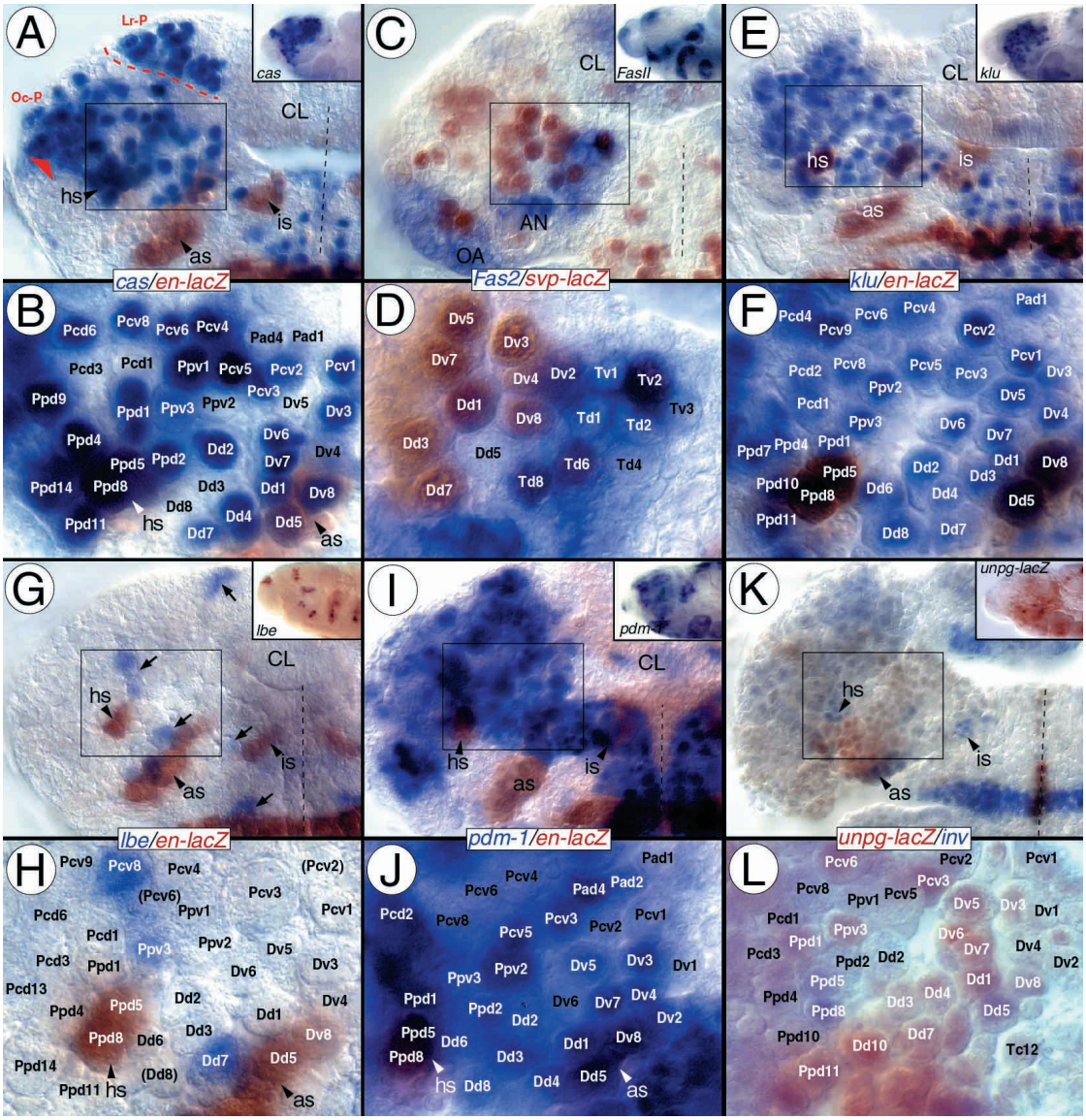

Fig. 6. Expression of *cas*, *Fas2*, *klu*, *lbe*, *pdm1* and *unpg* at embryonic stage 11. Double staining for expression of En/Inv (*lacZ* or antibody staining) or *svp-lacZ* (C,D) and Castor (*cas*; A,B), Fasciclin 2 (*Fas2*; C,D), Klumpfuss (*klu*; E,F), Ladybird early (*lbe*; G,H), Pou-domain 1 (*pdm1*; I,J), and *unplugged-lacZ* (*unpg-lacZ*; K,L), respectively, as indicated. (A,C,E,G,I,K) Focus is on the outer procephalic ectoderm of flat preparations (dashed line indicates ventral midline); insets show lateral views of wholemounts. (B,D,F,H,J,L) Higher magnifications of regions indicated by frames in A,C,E,G,I,K at the level of deuto- and protocerebral NBs; positively stained NBs are indicated by white labels, negative ones by black labels; NBs in brackets are not in focus. (A) Red arrowhead indicates expression of Cas in the outer ectoderm; dashed red line indicates the boundary between the labral (Lr-P) and ocular part of the protocerebrum (Oc-P). Cas is prominently expressed in most brain NBs (B). (C,D) Fas2 is expressed on the surface of most tritocerebral NBs and a few deutocerebral NBs (D). (E,F) Nuclear Klu expression is detected in almost all brain NBs. (G,H) Lbe is segmentally expressed in the head (arrows). (I,J) Approximately one half of the brain NBs express *pdm-1*, including most deuto- and tritocerebral, as well as central ocular and part of the labral, NBs. (K,L) *unpg-lacZ* is strongly expressed in almost all deutocerebral NBs, and in some ocular NBs close to the deutocerebral/ocular border. AN, antennal, segment; CL, clypeolabrum; Lr-Pc, labral part of the protocerebrum; Oc-Pc, ocular part of the protocerebrum; OA, Bolwig organ/optic lobe anlagen; as, *en* antennal stripe; hs, *en* head spot; is, *en* intercalary stripe. Anterior (a) is towards the top; dorsal (d) is towards the left.

NBs expression of Toy protein is transient and ceases during stage 11 (e.g. Dd3). To determine whether both *Pax6* genes, *ey* and *toy*, are co-expressed in identified NBs, we performed *toy* in situ hybridisation combined with an Ey antibody staining (data not shown). Interestingly, *toy* appears to be expressed only in those Ey-positive NBs deriving from the ocular segment; a co-expression was not seen in Ey-positive NBs of the trito- and deutocerebrum, or in Pad7 (Fig. 4A-C). During later embryogenesis, Toy protein is expressed in cells of the proto- and deutocerebrum, as well as in the tritocerebrum, although we did not identify any Toy-positive tritocerebral NB (Fig. 4C; and data not shown).

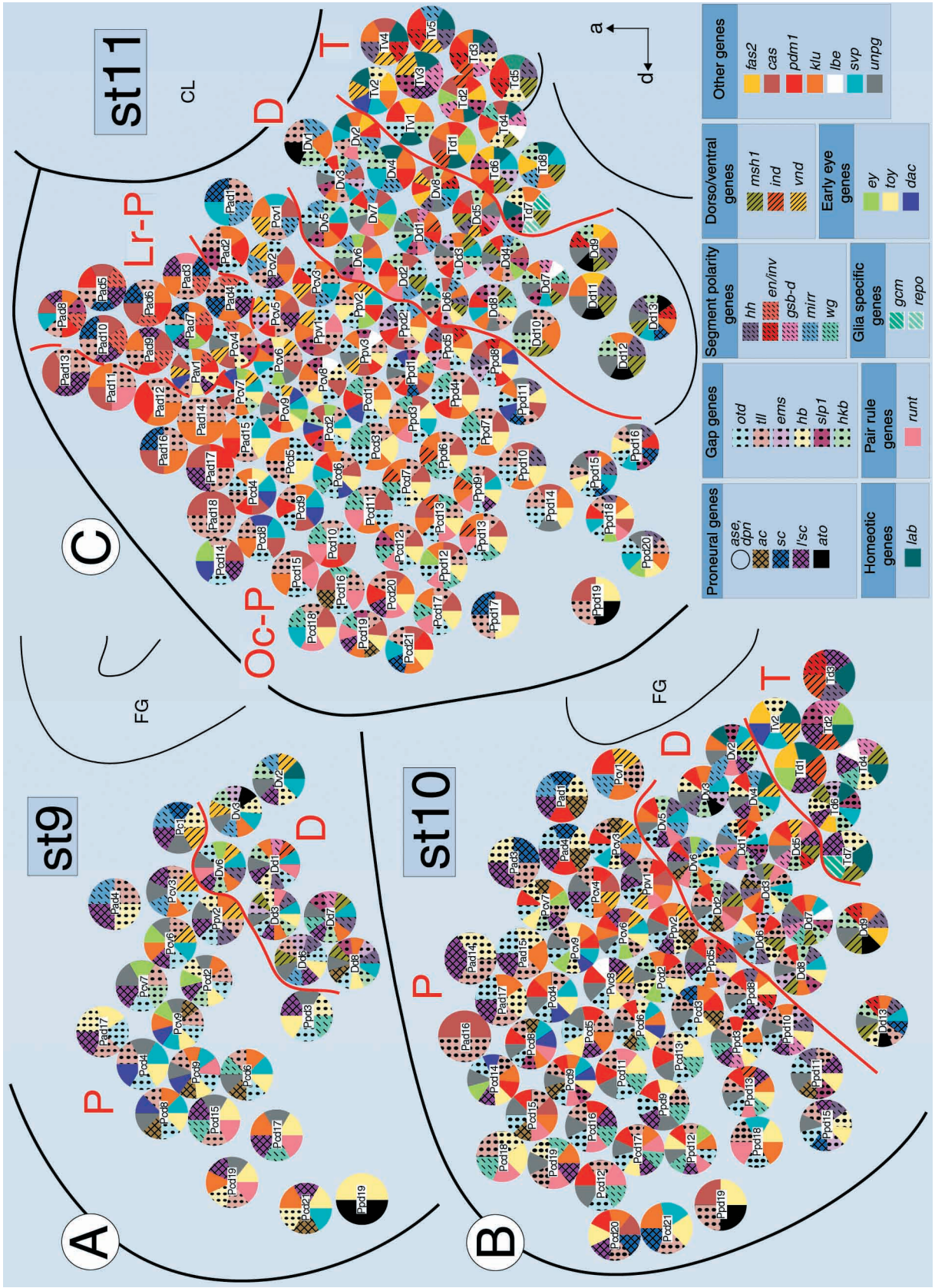
st9
st10
st11
A
B
C
P
D
T
FG
CL
Lr-P
Oc-P
a
d
Proneural genes
ase, dpn
ac
sc
lsc
ato
Gap genes
otd
tll
ems
hb
slp1
hkb
Segment polarity genes
hh
en/inv
gsb-d
mirr
wg
Dorso/ventral genes
msh1
ind
vnd
Early eye genes
ey
toy
dac
Glia specific genes
gcm
repo
Other genes
fas2
cas
pdm1
klu
lbe
svp
unpg
Homeotic genes
lab
Pair rule genes
runt

Fig. 7. Summary diagram of molecular markers expressed in brain NBs at different stages of embryonic development. Colour code indicates the expression of more than 40 different molecular markers (representing 34 different genes as listed) in individual brain NBs. In addition to the markers described in this paper, these maps include the expression of the proneural, segment polarity, DV patterning and glial marker genes, a detailed description of which is given in the accompanying papers (Urbach et al., 2003; Urbach and Technau, 2003). Each brain NB expresses a specific combination of molecular markers. Accordingly, each NB can be uniquely identified at stage 9 (A; st9), stage 10 (B; st10) and stage 11 (C; st11, which is when the entire population of brain NBs is established). Red lines indicate the neuromeric boundaries between the trito- (T) and deutocerebrum (D), and the ocular (Oc-P) and labral protocerebrum (Lr-P). Anterior (a) is towards the top; dorsal (d) is towards the left. CL, clypeolabrum; FG, foregut.

Further marker genes expressed in the head neuroectoderm and subsets of brain NBs

In order to establish further molecular markers that are specifically expressed in subsets of brain NBs, we investigated the expression of *castor*, *Fasciclin 2*, *klumpfuss*, *ladybird early*, *POU-domain 1 gene*, *runt* and *unplugged*. For details of the spatiotemporal expression pattern of these genes in the neuroectoderm and brain NBs (stages 9, 10 and 11), we refer to Figs 5-7 and Table 1.

In the trunk, the pair-rule gene *runt* is expressed in segmental domains of the ventral neuroectoderm (Gergen and Butler, 1988; Kania et al., 1990) (R.U. and G.M.T., unpublished) and in five NBs of row 2 and 3 and two NBs of row 5 (Dormand and Brand, 1998). Runt has also been shown to be expressed in an anterodorsal region of the blastoderm, corresponding to the presumptive head region (Gergen and Butler, 1988). En/Runt antibody co-labelling reveals that this Runt domain contributes to the ocular segment (Fig. 3B). In addition to the ocular segment, we find patches of *runt* expression in the intercalary, antennal and clypeolabral ectoderm (Fig. 3B,D,F), and in subsets of protocerebral and deutocerebral NBs (Fig. 3A,C,E,J). At stage 11, the protein is expressed in a total of 23 brain NBs (Fig. 3E), some of which initiate Runt expression after delamination from Runt-negative ectoderm, and in a large number of postmitotic cells until the end of embryogenesis (data not shown).

In the ventral nerve cord *castor* (*cas*, previously known as *ming*), encoding a zinc-finger protein, has been shown to be expressed in 18 NBs per hemineuromere (Doe, 1992), including early (S1-S2) and late delaminating (S3-S5) NBs, and to be involved in cell fate control within NB lineages (Cui and Doe, 1992; Mellerick et al., 1992). In the procephalon, *cas* expression is not detectable before stage10. It is dynamically expressed in the central and dorsal neuroectoderm of the ocular segment, in the median antennal segment, and, by stage 11, in the labral segment (Fig. 5F,I; Fig. 6A), which is surprising as *cas* is not expressed in the neuroectoderm of the trunk (Cui and Doe, 1992; Kambadur et al., 1998; Mellerick et al., 1992). A proportion of Cas-positive protocerebral and deutocerebral NBs derive from these domains. Most NBs appear to delaminate from Cas-negative neuroectoderm (Fig. 5D,G), and start to express *cas* at the time of formation, or show a reproducible delay in the onset of *cas* expression. The latter may already have produced a part of their lineage, which likewise has been proposed for early trunk NBs (e.g. NB7-4) (Cui and Doe, 1992). At late stage 11, Cas is expressed in about 60% of the total number of identified brain NBs (Fig. 5G, Fig. 6B).

Using an antibody against the cell membrane glycoprotein Fasciclin 2 (Fas2) (Grenningloh et al., 1991), we find that in the procephalic region Fas2 is first expressed by late stage 10 in an ectodermal patch at the border between the intercalary and antennal segment (Fig. 5E). Later it also covers the posterodorsal ocular neuroectoderm (including the optic lobe anlage) (Schmucker et al., 1997) and part of the labral ectoderm (Fig. 5H, Fig. 6C). Fas2 is also detected in brain NBs emerging from the antennal and intercalary neuroectoderm (Fig. 5D,G, Fig. 6D), and at a low level in a few dorsal ocular NBs (Fig. 5G,H). It has been found that Fas2 controls proneural gene activity in the eye/antennal imaginal disc (Garcia-Alonso et al., 1995), raising the possibility that it functions likewise in the procephalic neuroectoderm. However, we find that Fas2 expression in almost all identified brain NBs is initiated after delamination from Fas2-negative neuroectoderm, suggesting that Fas2 in the procephalic neuroectoderm is not involved in the regulation of proneural genes. It has been shown that Fas2 appears on the surface of neural somata prior to axon outgrowth; these neurones belong to ‘fibre tract founder clusters’ that pioneer the main axonal tracts in the brain (Nassif et al., 1998). Considering position and time point of development, we suggest that the identified Fas2-positive deuto- and tritocerebral NBs (Tv1, Tv2, Td1, Td2, Td6, Td8; Dv2, Dd9, Dd11) are the precursors of the ‘D/T fibre tract founder cluster’ (Nassif et al., 1998).

In the trunk, the zinc-finger transcription factor Klumpfuss (Klu) is expressed from stage 10 onwards in an increasing number of NBs, and at stage 11, almost all NBs (except NB2-3 and NB6-4) show nuclear Klu staining (Yang et al., 1997). We analysed the expression of Klu in the procephalon using an antibody against Klu and the P-*lacZ* enhancer trap strain *klu P212* (Yang et al., 1997) which basically show an identical expression pattern. Klu is not expressed in the neuroectoderm. Similar to the situation in the trunk CNS, we first find Klu protein at a detectable level at stage 9, in a subset of (about 17) brain NBs (Fig. 5A) and at late stage 11 in almost all brain NBs (Fig. 5G, Fig. 6E,F). For most NBs, there is a significant delay between birth and onset of *klu* expression. Klu also appears to be expressed in ganglion mother cells, as was shown for the trunk (Yang et al., 1997).

ladybird (*lb*), a tandem of the homeobox genes *ladybird early* (*lbe*) and *ladybird late* (*lbl*), both of which encode transcription factors, show a similar expression pattern, with *lbe* activity slightly preceding that of *lbl* (Jagla et al., 1994). At stage 11, both genes are expressed in segmental repetitive patches in the laterodorsal trunk ectoderm (Jagla et al., 1997) and specifically in one NB per hemineuromere, the lateral NB 5-6 (J. Urban, personal communication). Using an antibody against Lbe (Jagla et al., 1997) we first observed the protein by stage 10 in three small procephalic patches in the labral, ocular and antennal ectoderm (Fig. 5E), and at stage 11 in an additional patch of the intercalary ectoderm (Fig. 5H, Fig. 6G). Lbe is selectively expressed in only four brain NBs on either side: one in the tritocerebrum (Td4), one in the deutocerebrum (Dd7) and two in the protocerebrum (Ppv3, Pcv8; Fig. 5D,G, Fig. 6H). Wg/Lbe double labelling demonstrates that Lbe and Wg expression are colocalized in the intercalary, antennal and labral ectoderm, and in Td4 and Dd7; remarkably, the ocular Lbe-positive domain

and corresponding NBs (Ppv3 and Pcv8) are Wg negative [see Fig. 2G,H by Urbach and Technau (Urbach and Technau, 2003)]. Lbe protein is detected in the progeny of the identified brain NBs until the end of embryogenesis (data not shown).

The two closely related *Drosophila* POU-domain genes, *pdm1* (*nub* – FlyBase) and *pdm2*, are co-expressed in the developing CNS (before stage 13) and have been shown (at least with respect to the specification of the first ganglion mother cell of the truncal NB4-2) to be functionally redundant (Dick et al., 1991; Yang et al., 1993; Yeo et al., 1995). *pdm1* is expressed in the trunk neuroectoderm during the first and second wave of NB segregation (stage 8/9), and transiently in most NBs at stage 10 and 11 (Dick et al., 1991; Kambadur et al., 1998). In the procephalon the expression of the Pdm1 protein is highly dynamic (Fig. 5C,D,F,G,I; Fig. 6I,J). Until stage 10, Pdm1 is roughly restricted to the neuroectoderm of the antennal and ocular segments (as confirmed by Pdm1/En double labelling; Fig. 5C,F). Later, it is also found in the intercalary and labral ectoderm (Fig. 5I). At stage 9, NBs derived from Pdm1-positive neuroectoderm appear to be Pdm1 negative (Fig. 5A,C) and initiate *pdm1* expression at stage 10 (Fig. 5D) or stage 11 (Fig. 5G). At late stage 11, approximately one half of the brain NBs (about 52 NBs) express *pdm1*, including most deuto- and tritocerebral NBs, as well as central ocular NBs and part of the labral NBs (Fig. 5G).

Expression of the homeodomain gene *unplugged* (*unpg*) in the trunk starts at stage 8 in the ventral midline (Chiang et al., 1995) and becomes detectable in NBs of the ventral nerve cord at late stage 11 (Doe, 1992). Using an *unpg-lacZ* line (1912) (Cui and Doe, 1995; Doe, 1992), we observed *unpg* expression in the head at stage 9 in a large domain encompassing the intercalary, antennal and most of the ocular ectoderm (Fig. 5B). Until stage 11, the expression is gradually lost in the intercalary ectoderm, but upregulated in the dorsal part of the antennal and adjacent ocular ectoderm (Fig. 5E,H, Fig. 6K). In contrast to trunk NBs, which have already divided several times before expressing *unpg* at late stage 11 (Cui and Doe, 1995), we find *unpg-lacZ* to be weakly expressed already at stage 9 in all deutocerebral and almost all protocerebral NBs (Fig. 5A). At late stage 11, it is strongly expressed in almost all deutocerebral NBs (except for some ventral ones), and in some ocular NBs close to the deutocerebral/ocular border (Fig. 5G, Fig. 6L). Until the end of embryogenesis, *unpg* expression is observed in the putative progeny cells of the *unpg-lacZ*-positive deuto- and protocerebral NBs (data not shown).

All embryonic brain neuroblasts are uniquely identified

For thoracic and abdominal segments, it has been previously shown that each NB acquires a unique identity, which corresponds to a particular position in the neuroectoderm and (upon delamination) in the subectodermal NB layer, to a certain time point of its delamination, and to the combination of genes expressed (Broadus et al., 1995; Doe, 1992; Hartenstein and Campos-Ortega, 1984). These descriptions have provided an important basis for the elucidation of mechanisms controlling cell fate specification during early neurogenesis in the trunk region. In contrast to the truncal CNS, in which the segmental organization is obvious and the composition of the neuromeres is almost identical, the brain neuromeres are much more diverse and complex. Accordingly, information on identified brain cells and their gene expression is hardly available so far, and thus essential tools for investigating the mechanisms underlying pattern formation and cell diversity in the brain are lacking.

In this and the preceding studies (Urbach et al., 2003; Urbach and Technau, 2003), we provide an array of more than 40 different molecular markers (enhancer trap lines, antibodies, mRNA probes) characterizing the expression of 34 different genes in the early procephalic neuroectoderm and in the brain NBs emerging from it. We show that these marker genes are expressed in specific subsets of more than 100 brain NBs on either side (as summarized in Fig. 7 and Table 1). Based on the expression of specific combinations of molecular markers, morphological criteria as well as time and position of NB formation (see accompanying papers), each NB of the developing embryonic brain is now uniquely identified. Furthermore, as we analysed the expression of these markers in the neuroectoderm and in the NBs during various developmental stages, the fate of the individual brain progenitor cells can be followed through early neurogenesis. The unambiguous identification of NBs is a prerequisite for future work on mechanisms that control the specification and lineages of individual brain NBs.

Implications for regulatory interactions among genes expressed in the procephalic neuroectoderm and identified neuroblasts

Work on early neurogenesis in the trunk has provided evidence that the set of genes expressed within a particular proneural cluster of ectodermal cells specifies the individual fate of the NB it gives rise to. This is especially the case for segment polarity genes (for a review, see Bhat, 1999) and DV patterning genes (for a review, see Skeath, 1999). We show that these and other genes are also expressed in specific domains of the procephalic neurogenic region before brain NBs delaminate (e.g. *wg*, *gsb-d*, *en*, *hh*, *msh*, *ind*, *vnd*, *hkb*, *ems* and *slp1*). This implies that these genes might likewise be required for providing positional information in the procephalic neuroectoderm and for subsequent specification of individual brain NBs. Furthermore, all of the molecular markers assayed are expressed in different but overlapping subsets of brain NBs, and some of them we also observed to be expressed in (part of) their progeny cells (e.g. *en*, *hh-lacZ*, *msh-lacZ*, *ey*, *ems*, *hkb-lacZ*, *lbe*). Co-expression might hint to regulatory interactions among these genes within the neuroectoderm, particular NBs, and their lineage. Thus, in addition to being useful as molecular markers, the linking of these markers to specific regions of the neuroectoderm and to identified NBs uncovers candidate genes and putative molecular interactions that might be part of the machinery leading to cell diversity in the brain.

Serially homologous neuroblasts in the brain and ventral nerve cord

A comparison of NB patterns between neuromeres of the brain, as well as between the brain and ventral nerve cord, allows us to address the question of whether serial homologies exist among NBs of the different segmental regions. Within the trunk, the Cartesian grid-like expression of segment polarity and DV patterning genes is almost identical in each hemisegment and, accordingly, NBs developing from corresponding ‘quadrants’ acquire the same fate (reviewed by Bhat, 1999; Skeath, 1999). Indications for serial homology of NBs between different segments come from similarities in the time of formation, the

relative position within the evolving NB pattern and absolute position within a hemisegment, the co-expression of specific molecular markers, and similarities between their lineages.

Some of the molecular markers analysed here for brain NBs have been previously mapped in trunk NBs (e.g. Doe, 1992; Broadus et al., 1995). In addition to these, we analysed the expression of many other genes in the brain (this work) as well as in the gnathal, thoracic and abdominal segments (R.U. and G.M.T., unpublished). Owing to the large number of markers analysed, we find expression patterns that are unique to particular NBs. Comparison of the combination of markers expressed in individual NBs as well as their relative position within the NB layer of each segment suggests that several NBs exist in the brain that are serially homologous to NBs in the ventral nerve cord (VNC). This mainly applies to the posterior brain (deuto- and tritocerebrum), which is less derived than the anterior brain (protocerebrum). For example, according to these criteria, NB5-6 in all abdominal, thoracic and gnathal neuromeres (R.U. and G.M.T., unpublished) would be serially homologous to Td4 in the tritocerebrum and to Dd7 in the deutocerebrum (Fig. 7). These NBs exhibit a similar posterodorsal position within the respective neuromer immediately anterior to the En-positive NBs, and are the only NBs which specifically co-express the following molecular markers: *lbe* (which is generally expressed in only one NB per hemisegment), *wg*, *gsb-d*, *slp1* (except Td4), *msh*, *cas*, *seven-up* (except Td4), *pdm1*, *klu* and *asense*. Furthermore, some of the daughter cells of Td4 and NB5-6 co-express *ladybird* and the glia-specific marker *reversed polarity* [see Fig. 4D by Urbach et al. (Urbach et al., 2003)]. The existence of serially homologous NBs is intriguing as the number of NBs in the tritocerebrum and deutocerebrum is considerably reduced, the timecourse of neurogenesis within the brain and VNC is different [especially in the tritocerebrum the development of NBs is significantly delayed; see Urbach et al. (Urbach et al., 2003)], and the development of head segments (and consequently of brain neuromeres) has been assumed to be differently regulated (for a review, see Jürgens and Hartenstein, 1993).

In the VNC, serially homologous NBs that express the same combination of molecular markers (Broadus et al., 1995; Doe, 1992) give rise to almost identical cell lineages (Bossing et al., 1996; Schmidt et al., 1997), suggesting that similar regulatory interactions take place during the development of these NBs and their cell lineages. However, some of the serially homologous VNC lineages have been shown to include a subset of progeny cells that specifically differ between thoracic and abdominal neuromeres (Udolph et al., 1993; Bossing et al., 1996; Schmidt et al., 1997). We expect such segment-specific differences to be even more pronounced among serially homologous lineages within the brain and between the brain and VNC. Differences in the combination of marker genes expressed by putative serially homologous NBs may point to candidate genes conferring segment-specific characteristics to their lineages. Thus, unravelling the lineages of serially homologous NBs and the genetic network that controls their development will help to elucidate how region-specific structural and functional diversity in the CNS evolves from a basic developmental ground state.

Conclusions

We provide a comprehensive description of the spatiotemporal expression pattern of a large number of marker genes in the procephalic neuroectoderm and the entire population of identified brain NBs. Each of these genes is expressed in a specific subset of brain NBs. Thus, on one hand these markers are useful for tracing the fate of particular NBs in different genetic backgrounds. On the other hand, each of these genes itself is a candidate factor involved in the formation, specification or further development of specific NBs. Furthermore, each individual NB expresses a specific combination of markers that could reflect potential regulatory interactions among these genes. Finally, many of these genes are also known to be expressed in the neuroectoderm and/or NBs of the trunk, and to play a role during formation of the ventral nerve cord. Thus, the clarification of their function during brain development and comparison with the situation in the trunk would help us to understand what makes the brain different from the truncal part of the CNS.

The detailed descriptions of NB formation, segmentation and marker gene expression presented in this and the accompanying papers (Urbach et al., 2003; Urbach and Technau, 2003) in itself provide new insight into principles of early patterning of the brain. However, they should be particularly useful as a basis for approaching the molecular mechanisms that control the generation of cell diversity in the brain, as they make it feasible to study mutant phenotypes and the effects of genetic and experimental manipulations on the level of identified brain NBs.

We thank Andreas Prokop and Ana Rogulja-Ortmann for comments on the manuscript; and Andrea Brand, Günter Brönner, Markus Busslinger, Bill Chia, David Cribbs, Thomas Czerny, Chris Doe, Emma Dormand, Walter Gehring, Marcos Gonzales-Gaitan, Krzysztof Jagla, Thomas Kaufman, Judith Lengyel, Sandra Leuzinger, Ward Odenwald, Heinrich Reichert, Harald Vässin, Uwe Walldorf, Yuh-Nung Yan, Xiaohang Yang and the Bloomington stock center for providing antibodies, cDNAs and fly strains. This work was supported by grants from the 'Deutsche Forschungsgemeinschaft' and the 'Verband der chemischen Industrie' to G.M.T.

REFERENCES

Bhat, K. M. (1999). Segment polarity genes in neuroblast formation and identity specification during *Drosophila* neurogenesis. *BioEssays* **21**, 472-485.

Bhat, K. M., van Beers, E. H. and Bhat, P. (2000). *Sloppy paired* acts as the downstream target of *wingless* in the *Drosophila* CNS and interaction between *sloppy paired* and *gooseberry* inhibits *sloppy paired* during neurogenesis. *Development* **127**, 655-665.

Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1992). *deadpan*, an essential pan-neural gene in *Drosophila*, encodes a helix- loop-helix protein similar to the *hairy* gene product. *Genes Dev.* **6**, 2137-2151.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of *Drosophila melanogaster*. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* **179**, 41-64.

Brand, M., Jarman, A. P., Jan, L. Y. and Jan, Y. N. (1993). *asense* is a *Drosophila* neural precursor gene and is capable of initiating sense organ formation. *Development* **119**, 1-17.

Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G. M. and Doe, C. Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. *Mech. Dev.* **53**, 393-402.

Brody, T. and Odenwald, W. F. (2000). Programmed transformations in neuroblast gene expression during *Drosophila* CNS lineage development. *Dev. Biol.* **226**, 34-44.

Brönner, G. and Jäckle, H. (1991). Control and function of terminal gap gene activity in the posterior pole region of the *Drosophila* embryo. *Mech. Dev.* **35**, 205-211.

Cadigan, K. M., Grossniklaus, U. and Gehring, W. J. (1994a). Functional

redundancy: the respective roles of the two *sloppy paired* genes in *Drosophila* segmentation. *Proc. Natl. Acad. Sci. USA* **91**, 6324-6328.

Cadigan, K. M., Grossniklaus, U. and Gehring, W. J. (1994b). Localized expression of *sloppy paired* protein maintains the polarity of *Drosophila* parasegments. *Genes Dev.* **8**, 899-913.

Campos-Ortega, J. A. and Hartenstein, V. (1997). *The Embryonic Development of* Drosophila melanogaster. Berlin, Heidelberg, New York: Springer Verlag.

Chiang, C., Young, K. E. and Beachy, P. A. (1995). Control of *Drosophila* tracheal branching by the novel homeodomain gene *unplugged*, a regulatory target for genes of the bithorax complex. *Development* **121**, 3901-3912.

Cribbs, D. L., Pultz, M. A., Johnson, D., Mazzulla, M. and Kaufman, T. C. (1992). Structural complexity and evolutionary conservation of the *Drosophila* homeotic gene *proboscipedia*. *EMBO J.* **11**, 1437-1449.

Cui, X. and Doe, C. Q. (1992). *ming* is expressed in neuroblast sublineages and regulates gene expression in the *Drosophila* central nervous system. *Development* **116**, 943-952.

Cui, X. and Doe, C. Q. (1995). The role of the cell cycle and cytokinesis in regulating neuroblast sublineage gene expression in the *Drosophila* CNS. *Development* **121**, 3233-3243.

Czerny, T., Halder, G., Kloter, U., Souabni, A., Gehring, W. J. and Busslinger, M. (1999). *twin of eyeless*, a second *Pax-6* gene of *Drosophila*, acts upstream of *eyeless* in the control of eye development. *Mol. Cell* **3**, 297-307.

Dalton, D., Chadwick, R. and McGinnis, W. (1989). Expression and embryonic function of *empty spiracles*: a *Drosophila* homeo box gene with two patterning functions on the anterior-posterior axis of the embryo. *Genes Dev.* **3**, 1940-1956.

Dick, T., Yang, X., Yeo, S. and Chia, W. (1991). Two closely linked *Drosophila* POU domain genes are expressed in neuroblasts and sensory elements. *Proc. Natl. Acad. Sci. USA* **88**, 7645-7649.

Diederich, R. J., Merrill, V., Pultz, M. A. and Kaufman, T. C. (1989). Isolation, structure, and expression of *labial*, a homeotic gene of the Antennapedia-Complex involved in *Drosophila* head development. *Genes Dev.* **3**, 399-414.

Diederich, R. J., Pattatucci, A. M. and Kaufman, T. C. (1991). Developmental and evolutionary implications of *labial*, *Deformed* and *engrailed* expression in the *Drosophila* head. *Development* **113**, 273-281.

Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development* **116**, 855-863.

Doe, C. Q. and Technau, G. M. (1993). Identification and cell lineage of individual neural precursors in the *Drosophila* CNS. *Trends Neurosci.* **16**, 510-514.

Dormand, E. L. and Brand, A. H. (1998). *Runt* determines cell fates in the *Drosophila* embryonic CNS. *Development* **125**, 1659-1667.

Finkelstein, R. and Perrimon, N. (1990). The *orthodenticle* gene is regulated by *bicoid* and *torso* and specifies *Drosophila* head development. *Nature* **346**, 485-488.

Gao, Q., Wang, Y. and Finkelstein, R. (1996). *Orthodenticle* regulation during embryonic head development in *Drosophila*. *Mech. Dev.* **56**, 3-15.

Garcia-Alonso, L., VanBerkum, M. F., Grenningloh, G., Schuster, C. and Goodman, C. S. (1995). *Fasciclin II* controls proneural gene expression in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **92**, 10501-10505.

Gergen, J. P. and Butler, B. A. (1988). Isolation of the *Drosophila* segmentation gene *runt* and analysis of its expression during embryogenesis. *Genes Dev.* **2**, 1179-1193.

Gorman, M. J. and Kaufman, T. C. (1995). Genetic analysis of embryonic cis-acting regulatory elements of the *Drosophila* homeotic gene *sex combs reduced*. *Genetics* **140**, 557-572.

Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: *Fasciclin II* functions as a neuronal recognition molecule. *Cell* **57**, 45-57.

Grossniklaus, U., Pearson, R. K. and Gehring, W. (1992). The *Drosophila sloppy paired* locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. *Genes Dev.* **6**, 1030-1051.

Grossniklaus, U., Cadigan, K. M. and Gehring, W. J. (1994). Three maternal coordinate systems cooperate in the patterning of the *Drosophila* head. *Development* **120**, 3155-3171.

Halder, G., Callaerts, P. and Gehring, W. J. (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* **267**, 1788-1792.

Hama, C., Ali, Z. and Kornberg, T. B. (1990). Region-specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. *Genes Dev.* **4**, 1079-1093.

Hartenstein, V. and Campos-Ortega, J. A. (1984). Early neurogenesis in wild-type *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **193**, 308-325.

Hartmann, B., Hirth, F., Walldorf, U. and Reichert, H. (2000). Expression, regulation and function of the homeobox gene *empty spiracles* in brain and ventral nerve cord development of *Drosophila*. *Mech. Dev.* **90**, 143-153.

Hirth, F., Therianos, S., Loop, T., Gehring, W. J., Reichert, H. and Furukubo-Tokunaga, K. (1995). Developmental defects in brain segmentation caused by mutations of the homeobox genes *orthodenticle* and *empty spiracles* in *Drosophila*. *Neuron* **15**, 769-778.

Hirth, F., Hartmann, B. and Reichert, H. (1998). Homeotic gene action in embryonic brain development of *Drosophila*. *Development* **125**, 1579-1589.

Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. (2001). *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* **106**, 511-521.

Jagla, K., Jagla, T., Heitzler, P., Dretzen, G., Bellard, F. and Bellard, M. (1997). *ladybird*, a tandem of homeobox genes that maintain late *wingless* expression in terminal and dorsal epidermis of the *Drosophila* embryo. *Development* **124**, 91-100.

Jagla, K., Stanceva, I., Dretzen, G., Bellard, F. and Bellard, M. (1994). A distinct class of homeodomain proteins is encoded by two sequentially expressed *Drosophila* genes from the 93D/E cluster. *Nucleic Acids Res.* **22**, 1202-1207.

Jimenez, F. and Campos-Ortega, J. A. (1990). Defective neuroblast commitment in mutants of the *achaete-scute* complex and adjacent genes of *Drosophila melanogaster*. *Neuron* **5**, 81-89.

Jürgens, G. and Hartenstein, V. (1993). The terminal regions of the body pattern. In *The Development of* Drosophila melanogaster (ed. C. M. Bate and A. Martinez-Arias), pp. 687-746. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S. J. and Odenwald, W. F. (1998). Regulation of POU genes by *castor* and *hunchback* establishes layered compartments in the *Drosophila* CNS. *Genes Dev.* **12**, 246-260.

Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., Gehring, W. J. and Reichert, H. (2001). Differential expression and function of the *Drosophila Pax6* genes *eyeless* and *twin of eyeless* in embryonic central nervous system development. *Mech. Dev.* **103**, 71-78.

Kania, M. A., Bonner, A. S., Duffy, J. B. and Gergen, J. P. (1990). The *Drosophila* segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. *Genes Dev.* **4**, 1701-1713.

Kurusu, M., Nagao, T., Walldorf, U., Flister, S., Gehring, W. J. and Furukubo-Tokunaga, K. (2000). Genetic control of development of the mushroom bodies, the associative learning centers in the *Drosophila* brain, by the *eyeless*, *twin of eyeless*, and *dachshund* genes. *Proc. Natl. Acad. Sci. USA* **97**, 2140-2144.

Mahaffey, J. W., Diederich, R. J. and Kaufman, T. C. (1989). Novel patterns of homeotic protein accumulation in the head of the *Drosophila* embryo. *Development* **105**, 167-174.

Mardon, G., Solomon, N. M. and Rubin, G. M. (1994). *dachshund* encodes a nuclear protein required for normal eye and leg development in *Drosophila*. *Development* **120**, 3473-3486.

Martini, S. R., Roman, G., Meuser, S., Mardon, G. and Davis, R. L. (2000). The retinal determination gene, *dachshund*, is required for mushroom body cell differentiation. *Development* **127**, 2663-2672.

McDonald, J. A. and Doe, C. Q. (1997). Establishing neuroblast-specific gene expression in the *Drosophila* CNS: *huckebein* is activated by Wingless and Hedgehog and repressed by Engrailed and Gooseberry. *Development* **124**, 1079-1087.

Mellerick, D. M., Kassis, J. A., Zhang, S. D. and Odenwald, W. F. (1992). *castor* encodes a novel zinc finger protein required for the development of a subset of CNS neurons in *Drosophila*. *Neuron* **9**, 789-803.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C. S. and Rubin, G. M. (1990). The *Drosophila seven-up* gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell* **60**, 211-224.

Nassif, C., Noveen, A. and Hartenstein, V. (1998). Embryonic development of the *Drosophila* brain. I. Pattern of pioneer tracts. *J. Comp. Neurol.* **402**, 10-31.

Noveen, A., Daniel, A. and Hartenstein, V. (2000). Early development of the *Drosophila* mushroom body: the roles of *eyeless* and *dachshund*. *Development* **127**, 3475-3488.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole mount *Drosophila* embryos and larvae using antibody probes. In *Methods in Cell Biology.* Drosophila melanogaster*: Practical Uses in Cell Biology*, Vol. 44 (ed. L. S. B. Goldstein and E. Fyrberg), pp. 445-487. New York: Academic Press.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

Plickert, G., Gajewski, M., Gehrke, G., Gausepohl, H., Schlossherr, J. and Ibrahim, H. (1997). Automated in situ detection (AISD) of biomolecules. *Dev. Genes Evol.* **207**, 362-367.

Prokop, A. and Technau, G. M. (1994). Early tagma-specific commitment of *Drosophila* CNS progenitor NB1-1. *Development* **120**, 2567-2578.

Pultz, M. A., Diederich, R. J., Cribbs, D. L. and Kaufman, T. C. (1988). The *proboscipedia* locus of the Antennapedia complex: a molecular and genetic analysis. *Genes Dev.* **2**, 901-920.

Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. J. (1994). Homology of the *eyeless* gene of *Drosophila* to the *small eye* gene in mice and *aniridia* in humans. *Science* **265**, 785-789.

Reichert, H. (2002). Conserved genetic mechanisms for embryonic brain patterning. *Int. J. Dev. Biol.* **46**, 81-87.

Rogers, B. T. and Kaufman, T. C. (1996). Structure of the insect head as revealed by the EN protein pattern in developing embryos. *Development* **122**, 3419-3432.

Rudolph, K. M., Liaw, G. J., Daniel, A., Green, P., Courey, A. J., Hartenstein, V. and Lengyel, J. A. (1997). Complex regulatory region mediating *tailless* expression in early embryonic patterning and brain development. *Development* **124**, 4297-4308.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J. and Technau, G. M. (1997). The embryonic central nervous system lineages of *Drosophila melanogaster*. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Dev. Biol.* **189**, 186-204.

Schmidt-Ott, U., Gonzalez-Gaitan, M., Jäckle, H. and Technau, G. M. (1994). Number, identity, and sequence of the *Drosophila* head segments as revealed by neural elements and their deletion patterns in mutants. *Proc. Natl. Acad. Sci. USA* **91**, 8363-8367.

Schmucker, D., Jäckle, H. and Gaul, U. (1997). Genetic analysis of the larval optic nerve projection in *Drosophila*. *Development* **124**, 937-948.

Shen, W. and Mardon, G. (1997). Ectopic eye development in *Drosophila* induced by directed *dachshund* expression. *Development* **124**, 45-52.

Skeath, J. B. (1999). At the nexus between pattern formation and cell-type specification: the generation of individual neuroblast fates in the *Drosophila* embryonic central nervous system. *BioEssays* **21**, 922-931.

Tautz, D., Lehmann, R., Schnürch, H., Schuh, R., Seifert, E., Kienlin, A., Jones, K. and Jäckle, H. (1987). Finger protein of novel structure encoded by *hunchback*, a second member of the gap class of *Drosophila* segmentation genes. *Nature* **327**, 383-389

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Udolph, G., Prokop, A., Bossing, T. and Technau, G. M. (1993). A common precursor for glia and neurons in the embryonic CNS of *Drosophila* gives rise to segment-specific lineage variants. *Development* **118**, 765-775.

Udolph, G., Lüer, K., Bossing, T. and Technau, G. M. (1995). Commitment of CNS progenitors along the dorsoventral axis of *Drosophila* neuroectoderm. *Science* **269**, 1278-1281.

Urbach, R., Schnabel, R. and Technau, G. M. (2003). The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in *Drosophila*. *Development* **130**, 3589-3606.

Urbach, R. and Technau, G. M. (2003). Segment polarity and D/V patterning gene expression reveals segmental organization of the *Drosophila* brain. *Development* **130**, 3607-3620.

Walldorf, U. and Gehring, W. J. (1992). *empty spiracles*, a gap gene containing a homeobox involved in *Drosophila* head development. *EMBO J.* **11**, 2247-2259.

Weigel, D., Jürgens, G., Klingler, M. and Jäckle, H. (1990). Two gap genes mediate maternal terminal pattern information in *Drosophila*. *Science* **248**, 495-498.

Wieschaus, E., Perrimon, N. and Finkelstein, R. (1992). *orthodenticle* activity is required for the development of medial structures in the larval and adult epidermis of *Drosophila*. *Development* **115**, 801-811.

Yang, X., Yeo, S., Dick, T. and Chia, W. (1993). The role of a *Drosophila* POU homeo domain gene in the specification of neural precursor cell identity in the developing embryonic central nervous system. *Genes Dev.* **7**, 504-516.

Yang, X., Bahri, S., Klein, T. and Chia, W. (1997). *Klumpfuss*, a putative *Drosophila* zinc finger transcription factor, acts to differentiate between the identities of two secondary precursor cells within one neuroblast lineage. *Genes Dev.* **11**, 1396-1408.

Yeo, S. L., Lloyd, A., Kozak, K., Dinh, A., Dick, T., Yang, X., Skonju, S. and Chia, W. (1995). On the functional overlap between two *Drosophila* POU homeo domain genes and the cell fate specification of a CNS neural precursor. *Genes Dev.* **9**, 1223-1236.

Younossi-Hartenstein, A., Green, P., Liaw, G. J., Rudolph, K., Lengyel, J. and Hartenstein, V. (1997). Control of early neurogenesis of the *Drosophila* brain by the head gap genes *tll*, *otd*, *ems*, and *btd*. *Dev. Biol.* **182**, 270-283.